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

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Abstract

Purpose

Ifosfamide (IFO), an alkylating anticancer drug, is clinically effective but limited by severe toxicities, notably encephalopathy and urotoxicity. Chronotherapy -optimizing drug delivery according to circadian timing- may improve its therapeutic index and reduce toxicity. This study investigated circadian variation in IFO-induced toxicities in mice.

Methods

A total of 160 male Swiss albino mice were synchronized to a 12:12 h light-dark cycle. In an earlier study, IFO chronotolerance assessed at LD50 (a lethal dose to 50 % of mice) showed circadian survival rhythms but high lethality, limiting organ-specific analyses. In this study, we used a sublethal dose LD30 of IFO (the dose lethal to 30% of mice) to allow detailed evaluation of tissue chronotoxicity while reducing lethality.
140 mice were treated at four circadian times (1, 7, 13, and 19 **H**ours **A**fter **L**ight **O**nset, HALO), while twenty controls received distilled water. Endpoints included hematology, hepatic enzymes, histopathology (brain, liver, kidney, bladder) and neurobehavioral performance (wire-hanging test).

Results

Significant circadian variation was observed across multiple endpoints. Survival **(Fig.1)** and organ injury **(Fig.3)** strongly dependent on dosing time. Administration at **7 HALO** led to the worst outcomes, with **~47% survival and severe multi-organ injury**, whereas **13 HALO** preserved survival **(~82%)** and **minimized organ injury**. This six-hour shift represented a critical therapeutic window. Importantly, in mice (nocturnal species), 13 HALO corresponds to one hour after the onset of darkness (active phase), whereas in humans (diurnal species), the comparable stage corresponds to one hour after light onset (active phase), thus optimal treatment-timing is **inverted between mice and humans**. Assuming that for human the light onset occurs around 5a.m., 13 HALO in mice corresponds physiologically to 1 HALO, therefore, the optimal IFO administration time would be around 6 a.m.
Toxicity is expected to increase from 1 HALO until 13 HALO.

In rodent, hematological toxicity exhibited strong rhythmicity (Leukopenia: WBC: $F(3,45)=52.4$, $p<0.001$ and lymphopenia: lymphocytes: $F(3,45)=61.8$, $p<0.001$), with maximal suppression at 7 and 19 HALO and relative preservation at 13 HALO. Hepatic toxicity was time-dependent AST ($F(3,45)=33.7$, $p<0.001$); ALT ($F(3,45)=28.5$, $p<0.001$). Histopathological lesions varied by organ (bladder: Chi-square (3)=14.8, p=0.002; liver: Chi-square (3)=18.2, p<0.001; brain: Chi-square (3)=9.7, p=0.045; kidney: Chi-square (3)=11.6, p=0.021). Neurobehavior impairment was also modulated by circadian-.timing.

Conclusion
IFO-induced encephalopathy and multi-organ toxicities follow robust circadian rhythms in mice. Administration at 13 HALO (beginning of the active phase) minimized organ injury and improved survival. These findings support circadian-based chronotherapy as a promising strategy to improve IFO safety and therapeutic index for further clinical investigation.

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.
- **Author contributions:** MMC designed the study and wrote the first draft of the manuscript. DD conducted experiments and collected data. NAB supervised the project and revised the manuscript. All authors approved the final version.
- **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 institutional guidelines for the care and use of laboratory animals.
  Ethical approval was obtained from the Faculty of Medicine, Monastir.

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Introduction

Ifosfamide (IFO) is an oxazaphosphorine alkylating agent introduced in the early 1970s as a structural analog of cyclophosphamide (CP) **(Zalupski & Baker, 1988; Williams & Wainer, 1999)**. Despite sharing many pharmacological properties with CP, IFO presents a distinct toxicity profile that has limited its clinical acceptance. It remains, however, widely used in the treatment of sarcomas, lymphomas, and germ cell tumors, with an established role in pediatric oncology protocols for osteosarcoma, Ewing sarcoma, and Wilms tumor **(Furlanut & Franceschi, 2003; Idle & Beyoğlu, 2023)**. The clinical utility of IFO has been compromised by its dose-limiting urotoxicity and neurotoxicity.

Hemorrhagic cystitis, caused by the urotoxic metabolite acrolein, represented the most severe complication until the introduction of mesna in 1979, which effectively reduced bladder damage **(Sannu et al., 2017)**.
However, **ifosfamide-induced encephalopathy (IIE)** remains unpredictable and potentially life-threatening, with symptoms such as confusion, somnolence, hallucinations, and coma, with no universally effective preventive or curative therapy **(Williams & Wainer, 1999; Idle & Beyoğlu, 2023)**. In addition, hepatotoxicity and hematologic suppression contribute to the drug's narrow therapeutic index **(Floyd et al., 2006)**. Chronotherapy, the timed administration of chemotherapy according to circadian rhythms, has demonstrated benefits in improving tolerability and efficacy of several anticancer agents, including cisplatin and CP **(Haus et al., 1974; Reinberg & Smolensky, 1982; Lévi, 2001)**. Circadian clocks regulate host susceptibility to xenobiotics through oscillations in detoxification enzymes, DNA repair capacity, and immune function **(Fu & Kettner, 2013; Lee et al., 2019)**. We have previously demonstrated that IFO toxicity exhibits circadian variation in mice, with a bimodal rhythm of tolerance.

Building on these findings, the present study focuses on the **chronotoxicity of IFO** by specifically evaluating its major target organs: the bladder, liver, brain, kidney and hematopoietic system. Using a DL30 dose at four circadian stages (1, 7, 13, and 19 **H**ours **A**fter **L**ight **O**nset, **HALO**), we assessed hematological parameters, hepatic enzymes, histopathological lesions and neurobehavioral coordination. The goal was to determine whether circadian timing could modulate the severity of IFO-induced toxicities and thereby provide a basis for chronotherapy as a potential strategy to improve safety, particularly regarding neurotoxicity.

Methods

Animals and synchronization

160 male Swiss Albino mice (SIPHAT, Tunis), 8–10 weeks old (mean body weight ≈25 g), were synchronized for three weeks under a 12:12 light–dark cycle (lights on at 07:00, temperature 22 ± 2 °C, with food and water ad libitum). Circadian synchronization was confirmed by circadian body temperature rhythms. **(Waterhouse et al., 2005, Fig. S1.)**

Experimental design and treatment

Mice were randomized into four treatment groups corresponding to 1, 7, 13, and 19 HALO (n = 30 each). Each received a single intraperitoneal injection of IFO at LD30 (dose lethal to 30% of mice, previously established by probit analysis).
Control groups (n = 5 per time point) received sterile water.

Results are presented as mean ± SEM. One-way ANOVA Tukey's post-hoc test was used for continuous endpoints (hematology, transaminases, wire test). Organ lesion scores were compared with the Kruskal–Wallis test. Survival across dosing times was compared with the log-rank (Mantel–Cox) Chi-square test.

Lethal doses were estimated **(Fig.1)** from cumulative mortality by probit regression**:** **LD50 ≈ 520 mg/kg** and **LD30 ≈ 450 mg/kg** (single i.p. dose).

Hematotoxicity evaluation (Fig.2)
Day 7 post-injection, surviving mice were sacrificed, and blood was collected. Complete blood counts included:

- White blood cells (WBC, $10^3/mm^3$)
- Platelets (PLT, $10^3/mm^3$)
- Red blood cells (RBC, 10^3 $/mm^3$)
- Lymphocytes ($10^3/mm^3$)

Hepatotoxicity evaluation (Fig.2)
Blood serum was separated by centrifugation and analyzed for hepatic transaminases:

- Aspartate transaminase (AST, IU/L)
- Alanine transaminase (ALT, IU/L)

Histopathology (Fig.3)
On day 7 post-injection, surviving mice were sacrificed. Organs (bladder, liver, kidneys and brain) were collected, fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 µm, stained with hematoxylin-eosin, and scored semi-quantitatively on a 0–5 by a pathologist.

Neurotoxicity functional test (wire test) (Fig.4)
Neurobehavioral performance was assessed at day 7 post-treatment using the wire-hanging test. Each mouse was placed on a horizontal wire (2 mm diameter, 40 cm length, 30 cm above a padded surface). The latency to fall (maximum 60 seconds) was recorded. Results were averaged per group.

Results

Synchronization (Fig. S1)
The animals were well synchronized to the light–dark cycle, as confirmed by the circadian rhythm of body temperature. Core body temperature is a well-established physiological marker of circadian synchronization **(Waterhouse et al., 2005)**.

Survival rate to LD30 (Fig.1)
Survival curves differed significantly by dosing time (log-rank Chi-square (3) =11.3, p=0.009): highest at **13 HALO (82%)**, lowest at **7 HALO (47%)**. We reported that with LD50 we observed more lethality and the time for observation was longer (14 days).

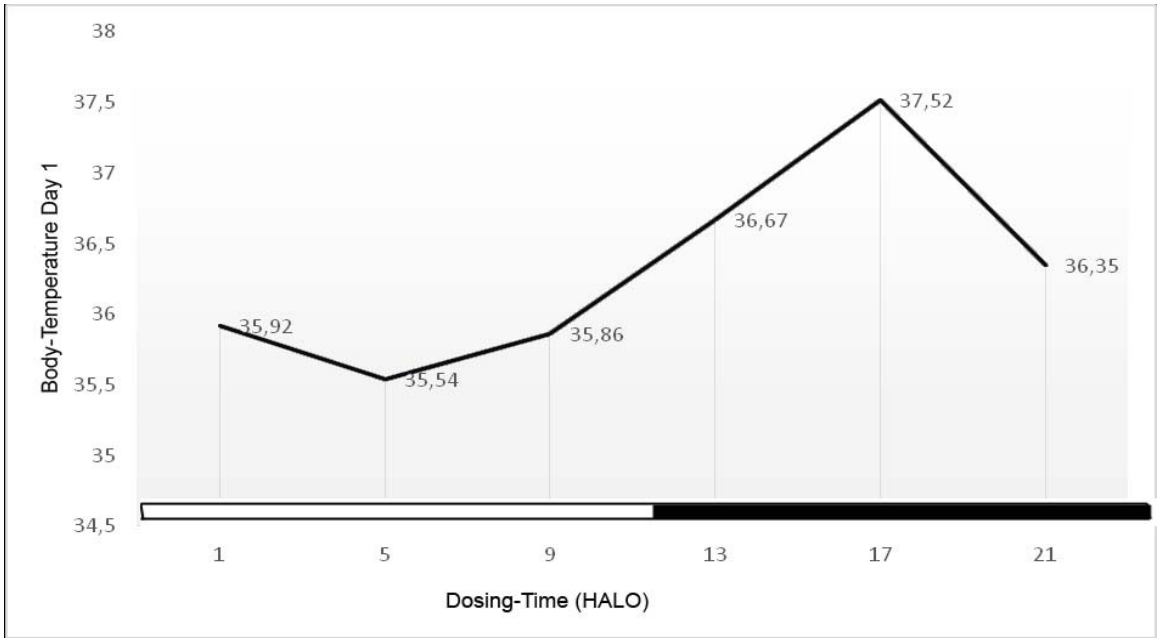

Fig. S1: Circadian variation of body temperature Day 1 (before treatment)
Body temperature rhythms confirmed circadian synchronization of mice before IFO administration.

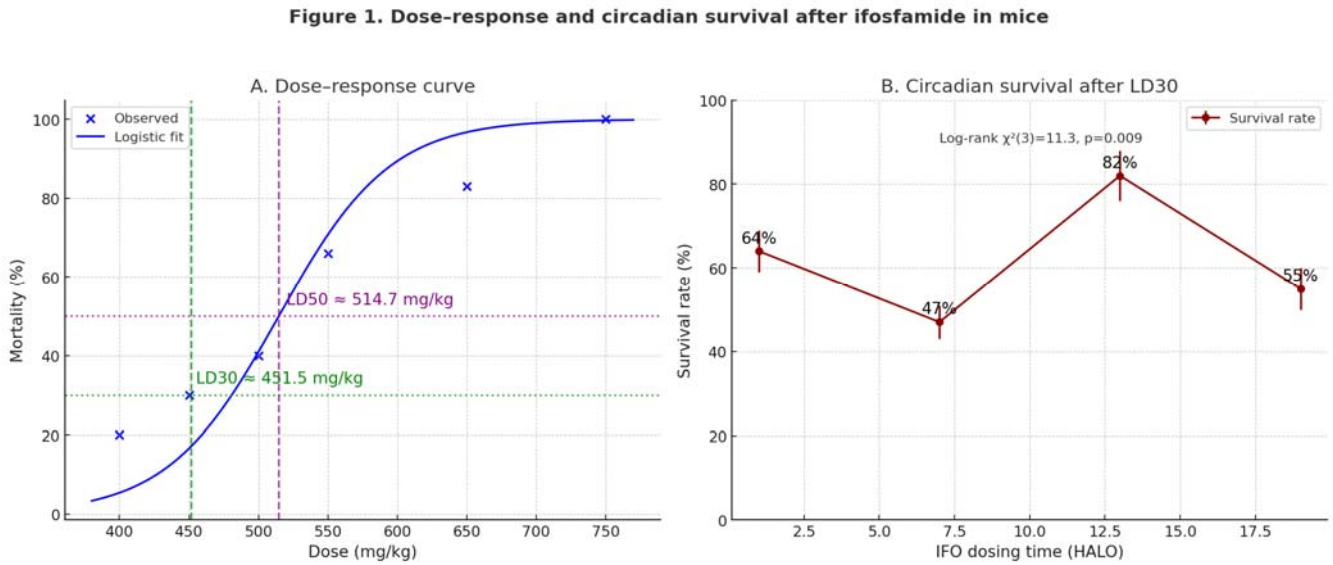

Fig. 1. Dose–response and circadian survival after ifosfamide in mice.
(A) Dose–response curve of ifosfamide–induced mortality in male Swiss albino mice. Mortality (%) increased in a dose-dependent manner, and probit analysis estimated an LD30 ≈ 450 mg/kg and an LD50 ≈ 520 mg/kg. (B) Circadian variation in survival following a single LD30 dose (450 mg/kg i.p.) administered at four circadian stages (1, 7, 13, and 19 HALO). Survival differed significantly across dosing times (log-rank Chi-square (3) = 11.3, p = 0.009), with the highest survival observed at 13 HALO (82%) and the lowest at 7 HALO (47%).

Hematology (Fig.2)

WBC: strong time effect (ANOVA $F(3,45)=52.4$, $p<0.001$). Leukopenia was maximal at 7 HALO ($1.03\times10^3/mm^3$) and 19 HALO ($0.70\times10^3/mm^3$) vs controls ($13\times10^3/mm^3$ and $4.76\times10^3/mm^3$), and least at 13 HALO ($1.90\times10^3/mm^3$).

Lymphocytes: significant time effect (ANOVA $F(3,45)=61.8$, $p<0.001$) with nadirs at 7 and 19 HALO and partial preservation at 13 HALO.

RBC: significant time effect (ANOVA $F(3,45)=29.7$, $p<0.001$); the largest drop occurred at 19 HALO ($3.07\times10^3/mm^3$) vs control $6.06\times10^3/mm^3$.

Platelets: significant (ANOVA $F(3,45)=25.1$, $p<0.001$), yet values showed the characteristic pattern—thrombocytopenia at 7 HALO ($455\times10^3/mm^3$), partial recovery at 13 HALO ($683.6\times10^3/mm^3$), and thrombocytosis at 19 HALO ($874.6\times10^3/mm^3$).

Circadian variations in hematological toxicity were prominent. Severe leukopenia and thrombocytopenia occurred at 7 and 19 HALO, while the least impairment was observed at 13 HALO. These results are consistent with previous chronotherapy findings on cyclophosphamide, where host hematopoietic tolerance was strongly time-dependent **(Haus et al., 1974; Lévi, 2001)**. Since white blood cells and platelets follow circadian rhythms in proliferation and turnover, administration during periods of maximal bone marrow activity (rest phases) likely exacerbates cytotoxicity. Conversely, dosing at 13 HALO (active phase for mice) preserved hematopoietic function.

Hepatic toxicity (Fig.2)

AST: significant time effect (ANOVA $F(3,45)=33.7$, $p<0.001$); peaks at 7 HALO (975.25 IU/L) and 19 HALO (959.6 IU/L); lower levels at 1 and 13 HALO (894.2 and 887 IU/L).

ALT: significant time effect (ANOVA $F(3,45)=28.5$, $p<0.001$); highest at 19 HALO (136.8 IU/L), ~120 IU/L at 1 and 7 HALO, and lowest at 13 HALO (116.7 IU/L).

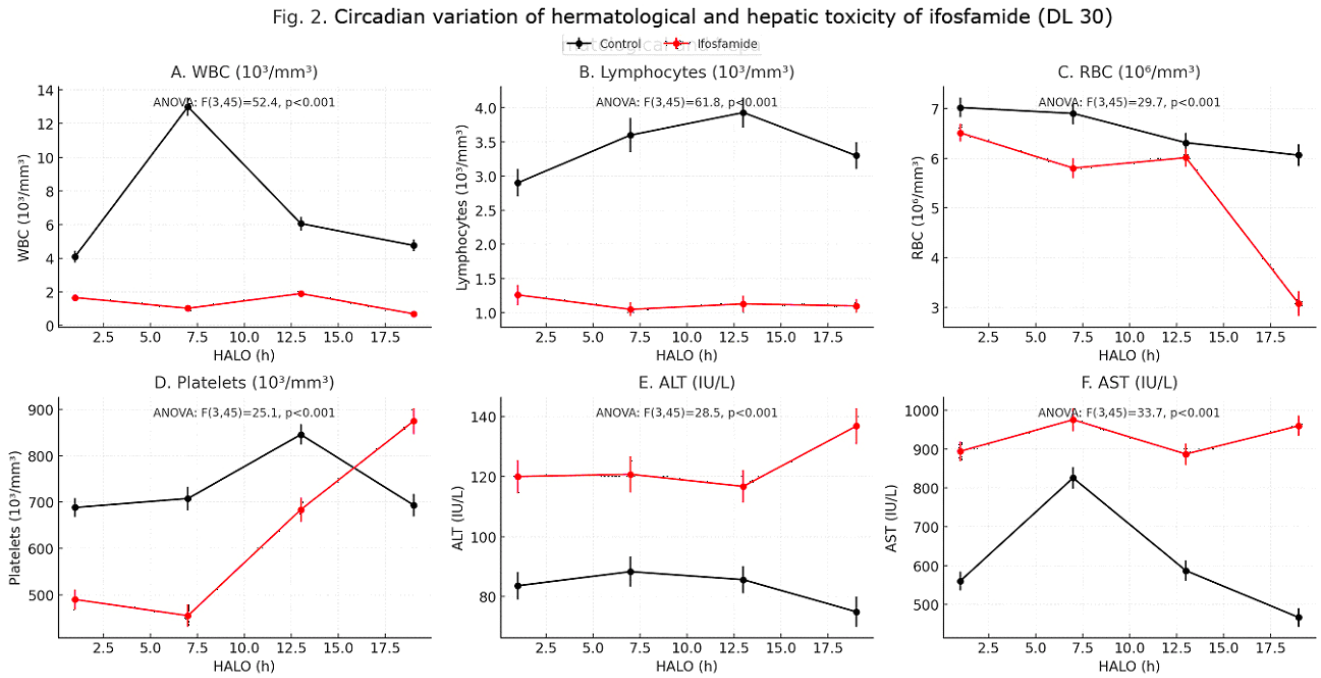

Fig. 2. Circadian variation of hematological and hepatic toxicities of ifosfamide (LD30).

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, $10^3/mm^3$): Ifosfamide induced a marked leukopenia at all dosing times, most pronounced at 7 and 19 HALO.

(B) Lymphocytes ($10^3/mm^3$): 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: $F(4,45)= 33.7$, $p < 0.001$).

Histopathology (semi-quantitative scores) (Fig.3)(Fig.S2, S3)
Bladder: Chi-square (3) =14.8, p=0.002**;** most severe at 19 HALO (score 3.5; necrosis/hemorrhage).
Liver: Chi-square (3) =18.2, p<0.001**;** highest at 7 HALO (5.66), lowest at 13 HALO (4.33).
Brain: Chi-square (3) =9.7, p=0.045; mild vacuolization/neuronal damage evident only at 19 HALO(1.25).
kidneys: Chi-square (3) = 11.6, *p* = 0.021

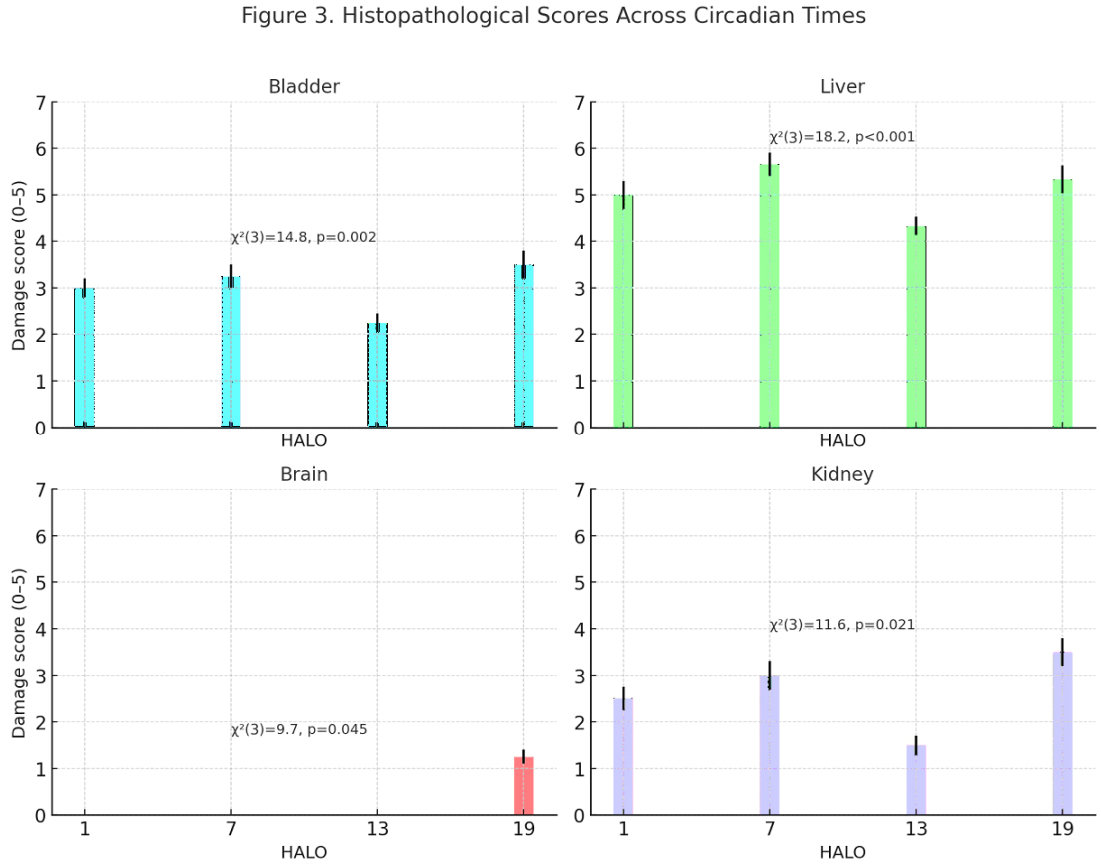

fig3. Circadian variation of ifosfamide-induced histopathological lesions in mice. Semi-quantitative damage scores (mean ± SEM, n = 6–8 per group) for bladder, liver, kidney, and brain across four dosing times (1, 7, 13, and 19 HALO). Lesion severity varied significantly with dosing time (Kruskal–Wallis:
(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.

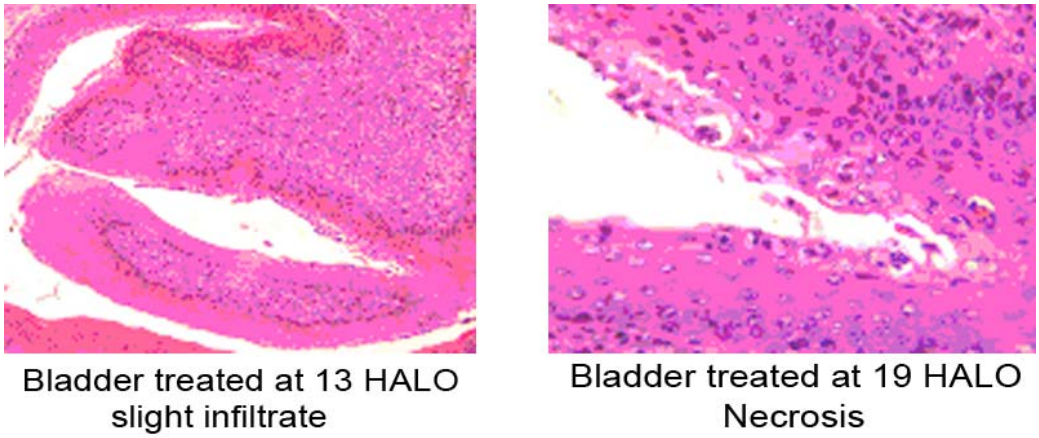

Fig.S2: **Representative histology of bladder: mild infiltrate at 13 HALO vs necrosis at 19 HALO.**

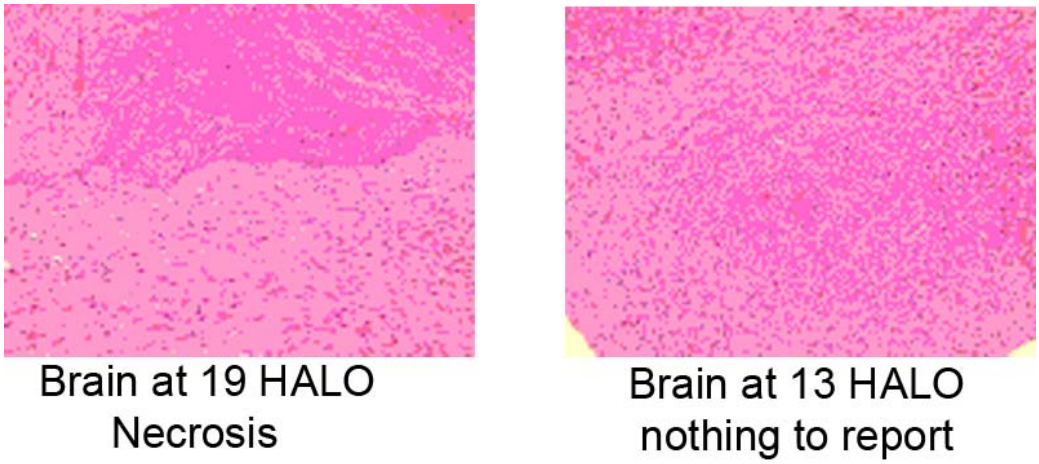

Fig.S3: **Representative histology of brain: necrosis at 19 HALO vs normal appearance at 13 HALO.**

Thus, histopathological evaluation confirmed biochemical data: the bladder and liver were the main targets of IFO toxicity, with worsening effects at 7 and 19 HALO. Transaminase levels (AST, ALT) and histopathology demonstrated significant circadian variation. Hepatic necrosis and enzyme elevation were maximal at 7 and 19 HALO, while 13 HALO showed partial protection. These findings support the concept that hepatic metabolism of IFO, mediated by cytochrome P450 isoenzymes, follows circadian fluctuations. During vulnerable phases, higher bioactivation of IFO into toxic metabolites (e.g., chloroacetaldehyde, acrolein) may explain increased hepatotoxicity.
Histological damage to the bladder epithelium was consistently severe at all dosing times, peaking at **19 HALO**. This confirms that the urotoxic metabolite acrolein is produced regardless of dosing time, though circadian modulation still influenced severity.
Mesna prophylaxis reduces but does not eliminate hemorrhagic cystitis **(Dechant et al., 1991; Sannu et al., 2017)**. Our results suggest that circadian dosing could complement pharmacological protection strategies.

Neurotoxicity (wire test) (Fig. 4)
Performance in the wire suspension test was significantly **(**one-way ANOVA, $p<0.01$**)** with the shortest latency to fall (≈12 s at 19 HALO), compared to **(≈40 s at 13 HALO)** and controls (≈55 s). Neurological impairment was minimal at 1 and 13 HALO. Neurotoxic effects were modest but measurable, with significant impairment in the wire suspension test at **19 HALO** and mild histological lesions in the brain. This aligns with the well-documented risk of ifosfamide-induced encephalopathy **(Williams & Wainer, 1999; Idle & Beyoğlu, 2023)**, a major limitation in its clinical use.
Circadian differences may reflect variation in blood-brain barrier permeability or in cerebral detoxification mechanisms (e.g., glutathione pathways). Importantly, dosing at **13 HALO** was associated with minimal neurological impairment, suggesting that chronotherapy could reduce encephalopathy incidence.

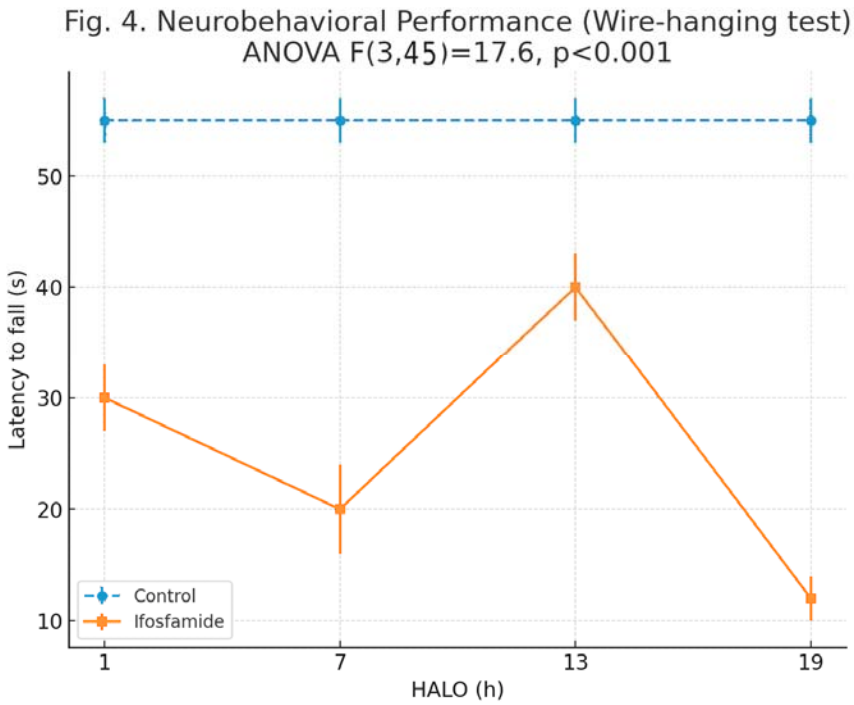

Fig. 4. Neurobehavioral performance in the wire-hanging test after ifosfamide administration (LD30) in mice.
Latency to fall (mean ± SEM, n = 6–8 per group) was recorded at 1, 7, 13, and 19 HALO. Ifosfamide-treated mice exhibited significant circadian variation in neuromuscular coordination (ANOVA $F(3,45) = 17.6$, $p < 0.001$). 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

This study demonstrates that ifosfamide (IFO) toxicity follows a clear circadian pattern. Hematopoietic suppression, hepatic enzyme elevations, urothelial lesions, and neurotoxicity were most pronounced at 7 and 19 HALO, while 13 HALO consistently provided protection. These findings align with prior chronotherapy studies on other oxazaphosphorine **(Haus et al., 1974; Reinberg & Smolensky, 1982 Lévi, 2001)**, suggesting that circadian fluctuations in bone marrow proliferation, hepatic cytochrome P450 activity, and blood–brain barrier permeability underlie these time-dependent toxicities.

Bladder injury occurred at all dosing times but peaked at 19 HALO, consistent with acrolein-mediated urotoxicity. While mesna reduces this complication, it does not abolish it, indicating that circadian dosing could provide complementary protection. Importantly, neurological impairment, reflecting ifosfamide-induced encephalopathy (IIE), was minimal at 13 HALO, suggesting that optimal timing of administration could mitigate this life-threatening adverse effect.

Clinical implications

Taken together, our results highlight that IFO toxicity is not only dose-dependent but also strongly time-dependent. The worst outcomes occurred at 7 and 19 HALO, whereas 13 HALO was consistently protective across multiple endpoints. For humans, this corresponds to the beginning of the daytime activity phase, reinforcing the rationale for early daytime administration in clinical protocols. Although mesna decreases urotoxicity and methylene blue may attenuate encephalopathy in some cases, no pharmacological strategy fully prevents IFO's toxicities. Circadian-based chronotherapy therefore represents a promising, non-pharmacological approach to improve tolerability, especially in pediatric oncology where IFO remains widely used.

Conclusion (Table 1)

This study confirms that ifosfamide exhibits strong circadian-dependent toxicity in mice. The best tolerance was observed at 13 HALO, corresponding to the early active/dark span in mice, which physiologically aligns with the early morning active span in humans (~6 a.m.). In contrast, administration during the late rest phase in humans (corresponding to 7 HALO in mice, ~12 p.m.) or late active phase (corresponding to 19 HALO in mice, ~2 p.m.) was consistently associated with higher toxicity.

IFO remains underexplored clinically due to its dose-limiting toxicities, particularly encephalopathy, an unpredictable complication without effective preventive therapy. Our findings support circadian-based scheduling as a strategy to mitigate general and organ-specific toxicities. Prospective clinical trials should evaluate whether early morning administration in humans can improve safety and therapeutic index.

HALO (Mice)	Survival after LD30 IFO (%)	Circadian phase (Mice)	Corresponding HALO (Humans)	Circadian phase (Humans)	Clinical implication
1 HALO	~64%	Early rest phase	13 HALO (~6 p.m.)	Early rest phase	Intermediate toxicity
7 HALO	~47%	Late-rest phase	19 HALO (~12 a.m.)	Late-rest phase	High toxicity
13 HALO	~82%	Early active phase	1 HALO (~6 a.m.)	Early active phase	Best tolerance
19 HALO	~55%	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, with survival after LD30 ifosfamide in mice.

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