



Recovery after a traumatic brain injury depends on diurnal variations Effect of cystatin C

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Abstract

Many studies indicate that the hour of the day at which the onset of stroke occurs is very important in patient recovery. Furthermore, multiple studies have been conducted which show that ischemia in rats produces different magnitudes of injury depending on the hour of the day at which it was induced. Using a traumatic brain injury (TBI) model, we analyzed the effect of the time of day on the recovery of rats and obtained a higher survival rate if TBI was induced at 01:00 h as compared with TBI induced at 13:00 h. We also analyzed the effect of the protease inhibitor cystatin C (CC) on the recovery of rats from TBI and found that it increased mortality and bleeding, and that these effects were more pronounced at 13:00 h.

Keywords: Cathepsins; Circadian rhythm; Brain vulnerability; Neuroprotection

Trauma to the brain represents one of the most important health problems in the world today. In USA, traumatic brain injury (TBI) accounts for nearly 40% of all deaths from acute injuries [12]. In Mexico, TBI is the fourth cause of mortality in the overall population and the first in the economically active segment of the population.

Trauma to the brain causes a variety of primary injuries via mechanical disruption of brain tissue and vasculature. It also causes cellular damage that develops over a period of hours or days after the initial traumatic brain insult. Such damage is known as secondary injury, and has been the target of several recent studies, because it is potentially reversible [14].

TBI triggers pathological pathways that may potentially harm brain cells. These mechanisms include excitotoxicity, formation of free radicals, inflammation, and apoptosis among others. However, auto-protective mechanisms are also induced by brain injury. These mediators possess damage-reducing properties and represent endogenous efforts to counteract traumatic damage and improve neuronal repair. Unfortunately, the secretion of such survival-promoting agents is limited both

in time and space and usually cannot completely counteract the overwhelming death-promoting processes mentioned above. However, identifying these mechanisms may permit the development of new therapeutic strategies that will more effectively mimic these protective effects.

The balance between harmful and protective mechanisms will ultimately determine the fate of the injured brain [3]. To that end, we analyzed the effect of the photoperiod on the recovery of rats subjected to TBI, since it has been documented that cerebral stroke susceptibility undergoes diurnal variations with a maximum risk peak within the first hours after awakening [4].

We also analyzed the neuroprotector role of cystatin C (CC) a protease inhibitor that inactivates cathepsins. Cathepsins are cysteine-proteases that seem to be released after traumatic injury and increase neuronal death [15]. CC release could be one of the auto-protective brain responses. We have previously found that CC concentration in cerebrospinal fluid varies with time of day, reaching higher values during the dark period and its minimal value at approximately 13:00 h [1]. We therefore explored the role of CC in the recovery of TBI applied at two different points of the light–dark cycle.

Male Wistar rats (250–300 g) were housed at constant ambient temperature maintained at 21 ± 1 °C and controlled

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light–dark cycle (lights-on from 08:00 to 20:00 h). Food and water were provided ad libitum.

A stainless steel cannula (23 gauge) was stereotactically implanted into the lateral ventricle according to the Paxinos and Watson Atlas [9] ($A = 0.8$, $L = 1.4$, $H = -3.8$). TBI was provoked at approximately $P = 4$. The entire procedure was performed while maintaining the rat under anesthesia. Fifteen minutes before applying the TBI, CC (0.348 pmol/4 μ l) or saline (4 μ l) was i.c.v. injected (1 μ l/min).

After 8 days of recovery, rats were housed individually, and a measured amount of food and water was delivered. After 24 h, rats were anesthetized with chloral hydrate (400 mg/kg) and a moderate head injury was induced at 01:00 or 13:00 h by dropping a weight (100 g \times 50 cm) onto the intact skull at $P = 4$. This model is known as closed head injury (CHI) [8]. Moderate-head injury is defined by injury resulting in a mortality rate of less than 40%. Given that CC concentration in cerebrospinal fluid showed higher values around 01:00 h and a minimal value was obtained at approximately 13:00 h, we selected these time points for our study. Sham control animals received anesthesia but no injury was applied.

After TBI, the following variables were evaluated: bleeding, food, and water intake for 24 h, neurological damage and injured area. Motor coordination was also evaluated before and after trauma.

We evaluated the external hemorrhage produced by the TBI, and compared the effects of CC administration versus control by weighing the blood drained after TBI. In brief, blood was drained and collected by pipette and then deposited into micro tubes and weighed.

We used 21-point behavioral–neurological scale reported by Hunter et al. [2] to evaluate neurological damage at days 1 and 8 after TBI. We evaluated paw placement (4 points), righting reflex (1), horizontal bar equilibrium (3), slanting platform (3), rotation (2), visual fore-paw reaching (2), contralateral reflex (2), motility (2), and general condition (2). Maximum score = 21. Although this scale was designed to evaluate damage caused by cerebral ischemia, it has been reported that these two models of brain damage share many similarities in displayed harmful pathways [3].

Motor coordination was measured by means of the Rotarod test (Ugo Basile, Italy), which consists of a rotating bar (\varnothing 5 cm)

driven by a motor at a 4 rpm speed. One day prior to TBI application, rats were trained to maintain their equilibrium on the test apparatus. The training consisted of three attempts to maintain the rat on the non-rotating apparatus and 10 subsequent 1-min attempts at 4 rpm. One day following TBI, rats were tested again on the Rotarod for 10 subsequent 1-min attempts.

Eight days after TBI, rats were perfused with 4% paraformaldehyde and the brains were removed, frozen, and sectioned (thickness: 30 μ m) in a cryostat. Brain sections were collected serially from bregma -0.92 to -5.8 at 300- μ m intervals from the injured area, and stained with cresyl-violet. Brains were classified according to the observed damage (0 = no damage; 3 = very large damage).

We observed a significant change in survival rates at 2, 24 h, and particularly at 8 days after TBI (Table 1). The time of day at which TBI was applied seemed to affect mortality at 2, 24 h, and 8 days with 12.5, 18.7, and 37.5% mortality, respectively, at 13:00 h versus no mortality when TBI was applied at 01:00 h ($*p < 0.025$, chi-square 6.1; 1 d.f.). CC administration increased mortality in both time-points of the day evaluated, particularly in the group receiving TBI at 13:00 h. Evaluation of mortality in this group after 2, 24 h and 8 days showed an increase of 35% in CC versus vehicle-treated rats. At 01:00 h, TBI induced mortality at a rate 9% greater in CC versus vehicle-treated rats after 1 and 8 days (Table 1).

We did not observe significant differences between the hemorrhages caused at 13:00 h and at 01:00 h by the TBI in vehicle-treated rats, but a marked difference with CC administration when TBI was applied at 13:00 h (Table 1).

We did not observe significant differences in food and water intake between treatments after TBI induced at 01:00 h, or in food intake when TBI was applied at 13:00 h. However, we observed null water intake in CC-treated rats after TBI at 13:00 h (Table 1).

We did not observe a significant effect of the hour of the day at which TBI was applied upon motor coordination or the neurological damage evaluated 24 h and 8 days after TBI (Fig. 1A and B). However, the CC-treated group displayed a worse Rotarod performance 24 h after TBI applied at 13:00 h (Fig. 1A).

Evaluation of brain damage area after TBI showed a significant increase in magnitude in the CC-treated group when TBI

Table 1
Data represent mean \pm S.E.

	13:00 h		01:00 h	
	Vehicle	CC	Vehicle	CC
Hemorrhage (g)	0.4 \pm 0.2 (n = 6)	4.2 \pm 1.9 ^a (n = 4)	0.9 \pm 0.5 (n = 4)	0.6 \pm 0.4 (n = 6)
Food intake (g)	2.1 \pm 0.8 (n = 10)	0.7 \pm 0.3 (n = 3)	2.6 \pm 0.8 (n = 13)	3.6 \pm 2.3 (n = 8)
Water intake	14.2 \pm 3.3 (n = 13)	0 ^b (n = 3)	12 \pm 4.7 (n = 13)	18 \pm 5.6 (n = 10)
Mortality after TBI ^c (%)				
<2 h	12.5	45.5	0	0
<24 h	18.7	54.5	0	9
<8 days	37.5	72.7	0	9

^a Statistically different from vehicle 13:00 ($p < 0.05$).

^b Statistically different from all ($p < 0.5$) one-way ANOVA.

^c $p > 0.05$ chi-square.

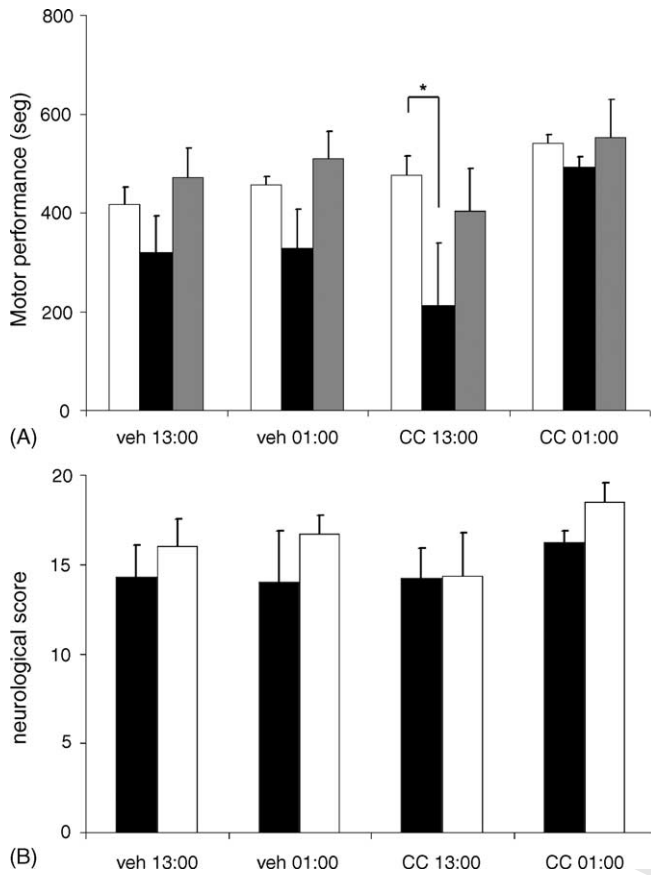


Fig. 1. (A) Time spent in Rotarod test. Bars represent mean + S.E.M. of the sum of 10 attempts in the Rotarod test before (white), 24 h (black) and 8 days after (grey) TBI. * $p < 0.05$ two-way ANOVA and Duncan test. (B) Neurological evaluation. Bars represent mean + S.E.M. of the neurological damage scale (Hunter et al. [2]) 1 (dark), and 8 (light) days after TBI; maximal score 21.

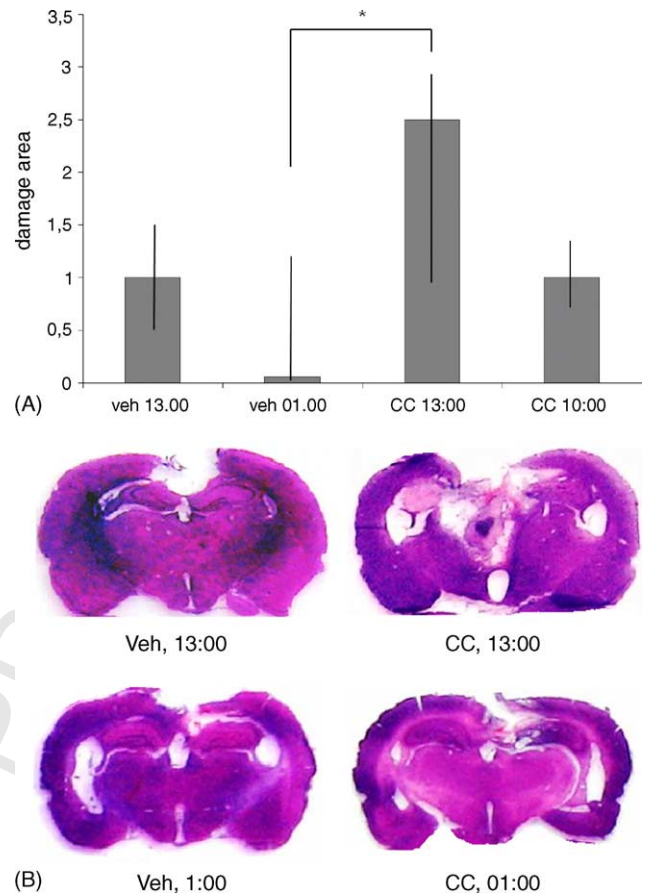


Fig. 2. (A) Brain-damage area. Data represent median, quartile 25 and 75 of the damage observed 8 days after TBI. (B) Brain sections stained with cresyl-violet. Representative sections are shown.

148 was applied at 13:00 h (Fig. 2A). The cresyl-violet stain revealed
 149 a larger area of damage in this group as shown in Fig. 2B.

150 Following cerebral damage, it has been shown that harmful
 151 mediators such as caspases and other types of proteases are
 152 released and cause secondary damage [10]. However, neuro-
 153 protective mediators are also released, and the balance between
 154 these classes of mediators determines the final outcome after
 155 injury [3]. We are interested in analyzing whether this balance
 156 between harmful and protective mechanism varies with time of
 157 day.

158 Our results indicate that the hour at which a subject suffers
 159 a TBI affects its life expectancy and recovery. The finding that
 160 brain vulnerability varies with time of day is not surprising; sev-
 161 eral chronobiologists have shown that almost every biological
 162 variable presents a circadian rhythm. Furthermore, several stud-
 163 ies have shown that the onset of brain stroke in humans occurs
 164 more frequently in the first hours of the day, between 06:00 and
 165 12:00 h, when a person normally awakens and begins his/her
 166 activities [4]. In rats, vessel occlusion causes a larger ischemic
 167 focus if induced in the darkness [14].

168 There are many factors that could explain the improved
 169 survival expectancy if the TBI is applied during the dark
 170 phase of the cycle. For example, it has recently been reported

that melatonin, which is higher during this phase [16], has a
 neuroprotective role [13].

We analyzed the variation of CC in the cerebrospinal fluid
 of rats over 24 h and we found an increase during the dark
 phase of the cycle [1]. We were therefore interested in studying
 whether CC could be one of the factors to explain our improved
 survival expectancy results if TBI is applied during the dark
 phase of the cycle. However, our results did not support our
 hypothesis that CC is a neuroprotective factor, at least not at the
 dosages employed in this study. There are contradictory reports
 about the role of CC as neuroprotector. For example, the inhi-
 bition of cathepsins B and L by CC seems to protect CA1 after
 global ischemia in primates [17,18], whereas intrahippocampal
 administration of CC in rats caused neuronal loss in the dentate
 granular cells layer [6].

Olson et al. [7] have shown that gene deletion of CC aggra-
 vates brain damage following focal ischemia but protects after
 global ischemia. We believe that a fine ratio between cathep-
 sins and CC must be maintained in order to achieve effective
 neuroprotection.

CC is a potent endogenous inhibitor of cysteine proteases,
 including cathepsin B, L, H, and S, which are involved in various
 biological processes, such as degradation of cellular proteins and
 regulation of pro-enzymes and pro-hormones [5].

We observed a non-significant difference in bleeding when TBI was applied at 01:00 or 13:00 h. However, a significant effect on bleeding was observed in the group receiving i.c.v. CC, although only in the group with TBI applied at 13:00 h. There are reports indicating that blood clotting time and coagulation factors present a circadian rhythm [11] and that several activated clotting factors are serine proteases but not cysteine proteases, which are inhibited by CC.

Regarding motor coordination and the neurological damage scale, we did not observe significant changes in the time of the day or in CC-treatment, and we attribute these findings to the fact that the corresponding test could only be made in surviving rats.

We conclude that the functional state of the brain varies with time of day; its vulnerability to damage seems to be greater during the light phase of the cycle.

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