Dosing-Time Dependence of Lethal Toxicity Induced by Nitroprusside in Young Mice

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Abstract

Sodium nitroprusside (SNP) is a potent vasodilator and is commonly used as an antihypertensive agent in postoperative cardiac surgical patients. In this study, we investigated whether the SNP-induced lethality is influenced by the dosing-time. Mortality was induced by administering SNP in different doses (4.9, 5.4, 6.0, 6.6 mg/kg, i.p.) to Swiss albino mice, 2 to 8 weeks old, synchronized for at least 2 weeks by 12 h light (rest)/12 h dark (activity) span. Each dose was administered to comparable groups of animals (n=6) at six different circadian times: 1, 5, 9, 13, 17, and 21 hours after light onset (HALO). Both χ² and cosinor methods were used to analyze the time series data. Statistically significant dosing time-dependent changes were validated in the daily scale with the males more sensitive than females. A ultradian (τ = 12 h) rhythm was detected by cosinor (P < 0.002) for each of the three administered doses (4.9, 5.4, 6.0 mg/kg) in 2 week old mice of both genders. Moreover, in addition to the ultradian rhythm, a significant (P < 0.004) circadian (τ = 24 h) component had been detected in 6 mg/kg SNP-treated male mice. However, there was only a circadian rhythm detected in 4 and 8 weeks old mice after acute SNP (6.0, 6.6 mg/kg) treatment, with peak time located at ≈ 21 and ≈ 17 HALO, respectively. In conclusion, tolerance to nitroprusside varies not only according to the circadian time but also according to the dose, age, and gender of animal.

Keywords: Acrophase; Age; Circadian rhythm; Mice; Utradian rhythm; NPS, Sodium nitroprusside

Introduction

Rhythmicity is a fundamental property of all living organisms. Biological rhythms are regular and periodic phenomena in living organisms. They can affect sensitivity to drugs, symptoms of various diseases and many other interactions between an organism and its environment. In most rodents and other mammals, rhythms are controlled and/or regulated by the suprachiasmatic nucleic [1-3]. Circadian rhythms are an evolutionary adaptation to day/night alternations, that is, an adaptation to the environmental changes caused by the Earth’s rotation. Circadian rhythms in both the desired and nondesired (toxic) effects of chemical and physical agents are known [4-7] and thus, the dosing of a medication at the proper biological time with reference to circadian rhythms can result in modulation of its toxicity [8-10].

Sodium nitroprusside (SNP), the potent vasodilator [11,12], is clinically used as an excellent drug for treating cardiac emergencies and lowering blood pressure [13-17]. However, as many drugs, toxic effects of SNP have been reported [18] and ascribed to various decomposition products such as nitric oxide, cyanide (CN), thiocyanate and nitrite. It’s generally agreed that SNP in humans and many other mammalian species undergoes in vivo to release free CN [19,20]. It has been shown in poisoned mice and rats that death after overdoses of SNP is a result of the accumulation of lethal levels of free CN [19]. The mechanism and site for this biotransformation reaction, however, have remained controversial. Under aerobic conditions in vitro SNP reacts with mammalian oxygenhemoglobin (HbO₂) in solution to generate the oxidized and unreacted HbO₂, the methemoglobin (MetHb). As a result of this reaction, the reduced SNP product is unstable and releases free CN. The lethal effects of CN have been known for more than a century and HCN, cyanide salts or cyonogenic compounds have been used in suicides, homicides and chemical warfare. Most of cyanide’s toxic effects have been attributed to histotoxic anoxia produced by reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain [21]. It’s generally believed that in vivo the primary pathway for CN detoxification is its metabolic oxidation to thiocyanate mainly catalysed by the mitochondrial enzyme rhodanese [22]. However, the toxic and lethal effects of CN are very rapid and treatment of its poisoning needs antidotes, which act very rapidly. Thus, the release of CN from SNP, a potent antihypertensive medicine particularly used in hypertension crises, during the course of therapy is also life-threatening problem [23, 24].

A major aim of chronotherapy is minimizing drug-induced side effects by a better optimization of drug at the adequate time-of-day. The purpose of this study was to assess the administration-time effects on SNP-induced lethality in 2, 4, and 8 week old mice.

Materials and Methods

Animals and synchronisation

Male and female Swiss albino mice of different age (2, 4, and 8 weeks) were used in this study. The animals were obtained from the Central Animal House (SIPHAT, 2013 Foundouck-Choucha, Tunisia), and experiments lasted from February to July 2007. They were

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acclimatized for at least 2 weeks prior to and during each study [25], to controlled conditions of 12 h light alternating with 12 h darkness (LD: 12:12), temperature (22 ± 2 °C), and relative humidity (50-60%). All animals were weaned at 21 days old and housed in groups of four or five in cages for each gender. During all experiments, standard food (ALMES, TN) and water were available ad libitum. Time series of the rectal temperature were used as marker rhythms to ensure the animals were (chrono-) physiologically healthy and synchronized to the LD entraining cycle. Time is expressed as hours after lights on (HALO).

Animals of each gender and age were divided randomly into various groups of six mice, each for using at one of six different circadian stages denoted as 2, 5, 9, 13, 17, and 21 HALO. This study was conducted at the FSB Toxicometry and Chronobiometry Laboratory, Bizerte, Tunisia in compliance with current Good Laboratory Practice standards and in accordance with current guidelines [26].

**Drug and lethal toxicity**

Nitroprusside ([Na₃Fe(CN)₆NO₂H₂O]) brown-red powder was kindly supplied by the Laboratory of Drug Control (1006 Tunis, Tunisia). The solution was freshly prepared each experiment day by adding an adequate volume of sterile distilled water to obtain the desired concentration. Four different doses of SNP were prepared following a geometric progression with a ratio of 1.1 between each two successive doses (4.9, 5.4, 6.0, and 6.6 mg/kg). Each mouse of each group (6 mice/time point) was treated once with a single lethal intraperitoneally (i.p.) dose of SNP. Different groups of 6 mice were injected at the six pre-selected different circadian stages. Three doses (4.9, 5.4, 6.0 mg/kg) were used for 2 week old mice, whereas 4-8 weeks old mice received two doses (6.0, 6.6 mg/kg). Lethality was recorded thereafter 1 h, and the mortality rate (estimated as the percentage of dead animals) was determined for each circadian time at the end of experiment.

**Statistical analysis**

In this study, time-point data (% of mortality rate) were computed as mean ± standard error of the mean (SEM) for each experiment. Differences in mortality rates were tested by the χ² method. Time series were analyzed by the cosinor method using trial periods (τ) of 24 and 12 h to determine the best fitting cosine function by the method of least squares [27]. For a given trial period, a rhythm is validated by the rejection of the hypothesis of which the amplitude is null (no rhythm [i.e., the rhythm’s amplitude = 0]). In principle, a rhythm is detected when A is different from zero (non-null amplitude as verified by the least squares [27]). For a given trial period, a rhythm is validated by the rejection of the hypothesis of which the amplitude is null (no rhythm).

**Results**

At each experiment, time series of rectal temperature were analyzed by cosinor test the day before treatment. This test revealed a significant (P < 0.0001) ultradian rhythm (with the first Φ = 2.8 HALO ± 36 min) in 2 week old mice. However, the cosinor detected significant (P < 0.0005) circadian rhythms both in 4 and 8 weeks old mice. As expected, the acrophase (Φ) was located in the first half of the dark (activity) span (Φ = 15.9 HALO ± 78 min) in young-grow-up mice (8 week old), while the Φ was detected nearly at the middle of light (rest) span (Φ = 5.2 HALO ± 90 min) in 4 week old young mice. These findings in rectal temperature confirmed the physiological synchronization of animals to the environmental LD cycle at the time of the SNP experiments.

The administration of SNP to mice caused a short period of calmness followed by increased respiration and convulsions, which started 3-6 min (depending on the dose) following SNP injection. The severity of convulsions was dependent on the dose of SNP, the

### Table 1: Parameters of ultradian and circadian lethality in 2 week old mice.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Gender</th>
<th>Period (h)</th>
<th>Mesor ± SEM (of lethality)</th>
<th>Amplitude ± SD (of lethality)</th>
<th>Acrophase ±SD (min)</th>
<th>P *</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.9</td>
<td>Male</td>
<td>12</td>
<td>61 ± 5</td>
<td>18 ± 17</td>
<td>9.0 ± 120</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>61 ± 5</td>
<td>12 ± 16</td>
<td>16.4 ± 74</td>
<td>NS</td>
</tr>
<tr>
<td>5.4</td>
<td>Female</td>
<td>12</td>
<td>40 ± 4</td>
<td>16 ± 15</td>
<td>9.8 ± 6.0</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>40 ± 4</td>
<td>14 ± 14</td>
<td>10.0 ± 60</td>
<td>NS</td>
</tr>
<tr>
<td>6.0</td>
<td>Female</td>
<td>24</td>
<td>69 ± 4</td>
<td>20 ± 13</td>
<td>9.1 ± 79</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>72 ± 4</td>
<td>15 ± 14</td>
<td>14.0 ± 258</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*From an F-test of the rejection of the null amplitude hypothesis.

*Time of maximum in fitted cosine function, in HALO.

NS: Not significant
higher the dose the stronger the convulsions. After SNP administration at lethal doses, in addition to the behavioural toxicity, we observed jumps, slow respiration, diving without breathing apparatus, brief and involuntary muscular contraction, stiffness of the tail, tonic paralysis of limbs, calmness, and death. The mean survival times of these mice depend both upon the time of day of SNP administration, and upon the dose of SNP administered. As expected, the greater the SNP dose the shorter the survival time. The mice that survived to lethal doses of SNP returned to a calm state after a period (20-45 min) of convulsions, depending on the dose. The number of dead animals was recorded 1 hour following SNP treatment. The 24-h mean mortality rate corresponds to the average of the mean values of the six dosing times. Mortality rates were dose-, gender-, and age-dependent ($\chi^2$ test, $P < 0.01$).

SNP-induced lethality was essentially similar regardless of the circadian time of administration. There were significant ($\chi^2$ test, $P < 0.0001$) differences between the different circadian dosing times in mortality rate. At the age of 2 weeks, statistically significant (Cosinor, $P < 0.001$) ultradian (12 h) rhythms were validated in male and female mice (Table 1). No rhythm was detected with the trial $\tau$ of 8 h. The first $\Phi$ of the 12 h rhythms (during the 24 h) occurred at $\approx$ 9-10.4 HALO ± 60 min (Table 1), with the subsequent peaks (during the 24 h) occurring at 9 HALO and 21 HALO (Figures 1 and 2). In addition, the young male mice treated with SNP 6 mg/kg exhibited a statistically significant circadian ($\tau = 24$ h; $P < 0.004$) rhythm with $\Phi = 14.0$ HALO ± 258 min (Table 1). Survival rates were greater ($\chi^2$ test, $P < 0.01$) in females than in males. The chronograms of morality rate at age 4 weeks (Figures 3-4 and Table 2) showed the curve patterns of the circadian rhythms validated by both $\chi^2$ test ($P \leq 0.0001$) and cosinor analysis ($P \leq 0.0005$). The circadian peak time was located at 21 HALO and the trough time at 9 HALO. The gender-related difference in mortality rates persisted until age 8 weeks. Figures 5-6 show the circadian rhythm patterns of lethal rate in young-grow-up mice of both genders. Peak and trough times are located at 17 and 5 HALO, respectively. Significant circadian rhythms (Table 3) were validated by $\chi^2$ test ($P < 0.0001$) and cosinor analysis ($P \leq 0.0007$).

**Discussion**

Chronobiological studies require animal synchronization. This is why, in our study, we used the L/D: 12/12 alternation as a synchronizer and temperature as a marker of circadian rhythms in mice. The present study revealed the existence of ultradian rhythms of rectal temperature in 2 week young mice. The detection of prominent ultradian rhythms
in the early stages of life in mammals, including man, is actually a common phenomenon [30-32]. In fact, the permanent presence of rhythmicity in rectal temperature from the birth in young mice revealed the existence of a particular rhythm genesis concerning this endogenous marker rhythm [33]. The data also demonstrate that the postnatal development of biological rhythms in mice proceeds gradually. Food intake represents a potent synchronizing cue for biological oscillations in adult animals. When restricted to a usual daytime, food intake may rapidly synchronize some daily oscillations independent of the suprachiasmatic nucleus (SCN) [34]. Restricted feeding and wheel running can even induce circadian rhythms that are not displayed during ad libitum feeding [35]. After delivery, the mouse mother nurses her newborns periodically, predominantly in her rest time, i.e., during the day, and leaves them so that she may feed herself during the night. The feeding regimen of the pups is thus mostly in opposite phase to those of adults. The partly restricted feeding of the suckling pups to the daytime might directly entrain the biological clock. Since the second week of life, the mouse pups open their eyes and start to change their feeding habits, i.e., to consume solid food (in addition to milk) during the night hours. After the time of weaning (21 days), until 4 weeks when pups gradually become independent of maternal care, the SCN controls rhythmic ad libitum feeding that mostly takes place during nighttimes.

To the best of our knowledge, little is known about the influence of circadian time on the toxicity of SNP [36]. The toxicity of SNP and its major metabolite, cyanide (CN−), was previously explored in non-chronobiological investigations in rodents and others mammals [37-39]. The subcutaneous administration of SNP to mice produced similar signs of toxicity described above, that were revealed by others authors when studying CN− toxicity in mice [39-40].

The purpose of the present study was to determine whether SNP tolerance varies over the course of the day, and if so, to investigate the potential influence of the age. Here, we expose dramatic time-of-day-dependent variations in SNP-induced mortality, which are also depending on the age of animal. Our findings revealed 12 h periodicities in the toxic effects of SNP in term of lethality rate in pre-weaned 2 week old mice. In one of our recent publications we [36] reported that the neurotoxic doses of SNP induced daily variations of ataxia with two peaks in young mice of the same age. The optimal circadian tolerance for SNP in term of survival rate was at 17 HALO with regard to the raw data depicted in the chronograms. However, at the neurotoxic, but nonlethal dose levels, SNP tolerance was observed at 9 HALO [36]. The difference in phases (by 8 h) of the two curve patterns indicates that the mechanisms involved in neurotoxicity might probably differ from those bound to the SNP-induced lethality. In fact, the distribution of SNP at a low and nonlethal dose through tissues would probably be
unimportant. It has been demonstrated that the neuronal toxicity might be related to SNP-induced oxidative effects [41]. On the other hand, the distribution becomes very important at lethal doses [19]. In that case, SNP is rapidly transformed in metabolites such as NO and CN. These last ones are then quickly biotransformed in the body to a much less toxic thiocyanate (SCN-) by the rhodanese [42, 43]. Cosinor analysis detected the presence of both a ultradian (12 h) period and circadian (24 h) component in 2 week old male mice treated with SNP 6 mg/kg. The ultradian period was found to be more prominent (i.e., had a larger amplitude and accounted for a greater percentage of variance) than the circadian period (Table 1). In addition, the large confidence limits (>2 h) of circadian acrophase (Φ = 14.0 HALO ± 258 min) and raw data (Figure 1) both confirm the fact that the 12 h period is considered as the “fundamental”. The coexistence of both circadian and ultradian rhythms in 6 mg/kg SNP-treated male mice can be explained by their high sensitivity to cyanide as compared to females [44]. These data suggested that, among other things, both dose and animal sensitivity may also influence the waveform of drug tolerance.

At the age of 4-8 weeks, circadian variations were found in the lethality rates of SNP with similarity in both genders (Figures 3, 4, 5, 13, 17, and 21 HALO) at various doses (6.0 and 6.6 mg/kg, i.p.) of sodium nitroprusside in 2 week old male (a) and female (b) mice. Each point represents the mean ± SEM of four independent experiments. The black bar corresponds to the dark period. Best drug tolerance occurred, irrespective to the gender, when the drug was injected in the light phase (during the animal’s rest span). The χ² test detected statistically significant differences in mortality rate related both to dosing time (P < 0.0001) and dose (P < 0.02).

Table 2: Parameters of ultradian and circadian lethality in 4 week old mice.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Gender</th>
<th>Period 1 (h)</th>
<th>Mesor ± SEM (% of lethality)</th>
<th>Amplitude ± SD (% of lethality)</th>
<th>Acrophase ± SSD (min)¹</th>
<th>P ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>Male</td>
<td>12</td>
<td>28 ± 5</td>
<td>10</td>
<td>11.9</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>28 ± 5</td>
<td>15 ± 14</td>
<td>21.4 ± 120</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12</td>
<td>25 ± 4</td>
<td>4</td>
<td>22.5 ± 120</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>26 ± 4</td>
<td>14 ± 13</td>
<td>20.9 ± 120</td>
<td>0.0005</td>
</tr>
<tr>
<td>6.6</td>
<td>Male</td>
<td>12</td>
<td>44 ± 4</td>
<td>4</td>
<td>0.5</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>44 ± 4</td>
<td>20 ± 13</td>
<td>18.7 ± 120</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12</td>
<td>36 ± 4</td>
<td>8</td>
<td>20.7 ± 120</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>35 ± 4</td>
<td>15 ± 14</td>
<td>12 ± 10</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹From an F test of the rejection of the null amplitude hypothesis.
²Time of maximum in fitted cosine function, in HALO.

NS: Not significant

Table 3: Parameters of ultradian and circadian lethality in 8 week old mice.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Gender</th>
<th>Period 1 (h)</th>
<th>Mesor ± SEM (% of lethality)</th>
<th>Amplitude ± SD (% of lethality)</th>
<th>Acrophase ± SSD (min)¹</th>
<th>P ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>Male</td>
<td>12</td>
<td>23 ± 4</td>
<td>6</td>
<td>1.8</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>25 ± 4</td>
<td>13 ± 12</td>
<td>18.8 ± 120</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12</td>
<td>16 ± 3</td>
<td>6</td>
<td>17.3 ± 120</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>15 ± 3</td>
<td>12 ± 10</td>
<td>4.2</td>
<td>0.0007</td>
</tr>
<tr>
<td>6.6</td>
<td>Male</td>
<td>12</td>
<td>33 ± 4</td>
<td>10</td>
<td>18.0 ± 120</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>32 ± 4</td>
<td>17 ± 13</td>
<td>4.8</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12</td>
<td>30 ± 4</td>
<td>10</td>
<td>18.7 ± 120</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>30 ± 4</td>
<td>15 ± 14</td>
<td>10</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹From an F test of the rejection of the null amplitude hypothesis.
²Time of maximum in fitted cosine function, in HALO.

NS: Not significant
as sulfur donors [50]. The high sensitivity of the young pre-weaned mice to SNP would be due to their less ability to mobilize thiols from stores despite increasing cyanide concentrations, leading to accelerated toxicity [51]. In contrast, normal young-weaned mice can detoxify SNP using existing (dietary provided) sulfur stores. Assuming normal rhodanese activity, onset of toxicity may be minutes to hour depending on the above-mentioned sulfur stores. However, there is considerable controversy regarding the true incidence of clinically significant cyanide toxicity. Thus, a correlation would be expected between the sensitivity of mice to lethal doses of SNP and rhodanese activity if the assertions concerning the role of this enzyme in the detoxication of CN are true. However, our previous study [52] showed that there was no correlation between the hepatic enzyme and SNP-induced mortality. These results indicate that neither hepatic rhodanese nor CN detoxification are determining in modelling of SNP-induced lethality. Therefore, a systematic search would be necessary for the true locus of that bionitrification reaction. At this stage, the question that arises is when SNP should be administered in the time scale to increase its efficiency while decreasing its toxicity.

The overall findings of this study suggest that SNP presents level doping-time (biological rhythm) dependent lethal toxicity. This must be considered when this medication is recommended to patients to manage the toxic side effects of the drug. In conclusion, based on the significant time-of-day effect, the results of this study favour a clinical optimization through SNP chronotherapy. In addition, significant age- and gender-related variations revealed in this study suggest that, in both clinical and scientific settings, appropriate comparison of toxicity level can only be achieved based on data obtained from animals of the same age and gender.

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Declaration of Interest Statement
The authors declare that they have no competing interests.

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