Circadian variation of antinociceptive effect of adenosine and adenosine A1 receptor agonists, N6-phenylisopropyl adenosine and 2-chloroadenosine, in mice

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Summary
In this research circadian variation of antinociceptive effect of adenosine and two A1 adenosine receptor agonists, (R) N6-phenylisopropyl adenosine **(**R-PIA**)** and 2-chloroadenosine (2-CAdo), and pain were studied in male mice that housed under control light phase for two weeks. Circadian variation of pain was performed on intact mice by using hot plate test. Doses of 40, 2 and 0.6 mg/kg of adenosine, 2-ClAdo and R-PIA, respectively were injected intraperitoneally to three separated groups of six male mice at six hour intervals (09:00, 15:00, 21:00, and 03:00). The control group received normal saline. The result of circadian rhythm of pain showed that the minimum nociceptive effect was observed in dark phase in mice and it was time dependent. The peak of antinociceptive effect of adenosine was in dark phase (21:00 P.M.) at 0.5 h after of injection. The results of 2-ClAdo indicated that maximum of antinociceptive effect was in dark phase and results of R-PIA exhibited that peak of antinociceptive activity was in light phase. This study indicated that the pattern of pain in male mice has circadian variation. The antinociceptive effect of adenosine and its two agonists was time dependent. This circadian variation in antinociceptive activity may be important in the administration of these agents.
Key words: adenosine, antinociceptive, pain, chronopharmacology, circadian variation, N6-phenylisopropyl adenosine and 2-chloroadenosine

Introduction
Chronopharmacology is the study of time dependent physiological rhythms to drug and its relationship to current drug therapy (1). Circadian rhythms are established at every level of eukaryotic organization nearly in all functions of the body like cardiovascular, pulmonary, hepatic and renal functions (2) that influencing pharmacokinetic factors, such as drug absorption, distribution, metabolism and renal elimination with significant daily variations (3, 4). Thus, biological rhythms may have an effect on drug therapy (2). The efficiency and toxicity of a lot of drugs depend on time of dose administration during the day under the control of circadian clock (5, 6).

Adenosine is an endogenous purine molecule released from different tissues that modulates a variety of biological responses by interaction with G-protein-coupled receptors (7). The adenosine receptors are termed purine P1 receptors that have been subdivided into four adenosine receptor subtypes (A1, A2A, A2B and A3) (6). Adenosine actions may be mediated pre- and postsynaptycally through receptor mediated mechanisms including second messenger systems, transmembrane ion fluxes and neurotransmitter release (8). In specific conditions, such as metabolic stress (9), pain, ischemia, hypoxia, trauma, seizures and inflammation the release of adenosine is increased due to an high energy demand of ATP, that is metabolized to AMP and adenosine (7,

10, 11). Adenosine also regulates pain transmission by actions at spinal, supraspinal and peripheral sites through the activation adenosine A1 receptors and may play an important role in inflammatory and neuropathic pain (12). Indeed, the antinociceptive effect of spinally administered adenosine A1 receptor agonists has been indicated to be mediated by both pre- and postsynaptic adenosine A1 receptors (6). The elevation of endogenous adenosine levels is insufficient to exert pharmacological effects because extracellular adenosine usually disappears quickly due to its rapid uptake into adjacent cells (e.g., erythrocytes and endothelial cells) and subsequent metabolism (7).

Previous studies showed that adenosine concentrations in different tissues such as brain, blood and liver of the rat had periodic changes. The rhythmicity of this molecule, as well as its metabolism and even the presence of specific receptors, suggests a regulatory role in eukaryotic cells and in multicellular organisms (13). In the brain the activity of adenosine-metabolizing enzymes are low during the day; therefore the high adenosine concentration was observed (14). In the blood, circadian variation with low levels of adenosine from 08.00 - 20.00 h, due to the high activity of adenosine-metabolizing enzymes followed by an increase in the level of inosine and hypoxanthine,was observed and then increased at night. In the liver, adenosine was high during the night while inosine and hypoxanthine remained low along the 24 hours (15). The circadian variation of the toxicity and nociceptive activity of aminophylline as an antagonist of adenosine receptor was demonstrated in our previous study (16). It has been considered that pain is seldom constant during the day; rather it is circadian-time dependent (17). Since adenosine is a neuroregulator of pain pathways and rhythmicity of this molecule has been demonstrated, thus, the evaluation of antinociceptive effect of adenosine and two A1 agonist receptors and relevance with time was studied in mice.

Materials and methods

Animals

Male albino mice (25–30 g) were obtained from a randomly bred colony maintained on special diet in the animal house of Mashhad University of Medical Sciences. Animals were housed in a colony room under a 12/12 h light/dark (07:00- 19:00) cycle at 21 ± 2 °C .The animals had free access to water and food. All animal experiments were carried out in accordance with Mashhad University of Medical Sciences, Ethical Committee Acts.

Preparation of materials

At first, the doses of adenosine, 2-ClAdo and R-PIA that have the best antinociceptive effect were determined by using hot-plate tests. Adenosine, R-Phenylisopropyladenosine (R-PIA) and 2-Chloroadenosine (2-ClAdo) were purchased from Sigma Chemical Co.

Circadian rhythm of Pain

Circadian variation of Pain was measured at 4 and 6 hours interval by using hot-plate test on intact mice. In hot plate, the temperature of the metal surface was maintained at 55 ± 0.2 °C. The latency to a discomfort reaction (licking paws or jumping) was determined before and after drug administration. The cut-off time was 25 sec (18).

Circadian rhythm of antinociceptive activity

Antinociceptive activity was assessed by using hot-plate test. Doses of 40, 2 and 0.6 mg/kg of adenosine, 2 ClAdo and R-PIA, respectively, that have the best antinociceptive effect were injected intraperitoneally to three separate groups of six male mice at six hour intervals (09:00, 15:00, 21:00, 03:00) and the control group received normal saline. These tests were done in 0, 0.5 and 1 h after of injection of adenosine and 0, 0.5, 1, 1.5 and 2 h after injection of 2 ClAdo and R-PIA, respectively.

Statistical analysis

The obtained data were expressed as mean values ± S.E.M. and tested with analysis of variance followed by the multiple comparison test of Tukey– Kramer. Discrepancies with $P < 0.05$ were considered significant. For determination the role of times after injection

and different times of day on pain, two way analysis of variance of ANOVA also was used.

Results

Circadian rhythm of Pain

The results of this experiment showed that practically the changes of pain rhythm were significant (09:00 vs 03:00, P<0.05) (Fig. 1).

Circadian rhythm of antinociceptive activity of adenosine

Two peak of antinociceptive effect (21:00 P.M and 9:00 A.M.) of adenosine were observed at 0.5 h after of injection (p<0.05) (Fig. 2).

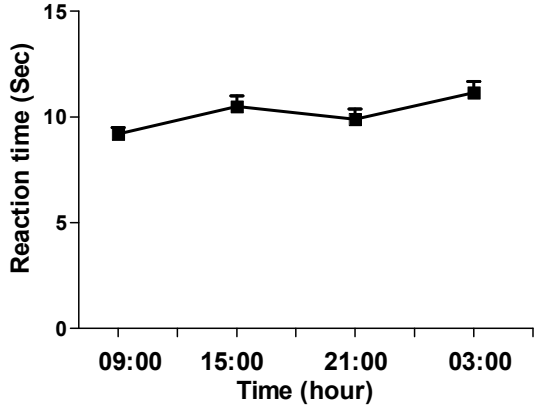

Figure.1. Circadian variation of the pain threshold on intact mice in the hot-plate test. Each point represents the mean ± S.E.M. of reaction time for *n=6* experiments on mice. There is significant difference between different times of day ($P > 0.05$, Tukey–Kramer test).

Circadian rhythm of antinociceptive activity of 2-CLAdo

The results of antinociceptive activity 2-CLAdo 1, 1.5 and 2 h after of injection indicated that the highest antinociceptive activity was seen in dark phase (21:00 vs 09:00 1 h after injection, p<0.05 and 21:00 vs 09:00 2 h after injection, p<0.01) (Fig. 3).

Circadian rhythm of antinociceptive activity of R-*PIA*

The results of antinociceptive activity R-PIA 1, 1.5 and 2 h after of injection indicated that the highest antinociceptive activity was seen in light phase (09:00 vs 21:00 1 and 1.5 h after injection, p<0.01 and 09:00 vs 21:00 2 h after injection, p<0.05) (Fig. 4).

Discussion

This study demonstrated that the pattern of pain in male mice has variation in day-night period, and this change was observed more at

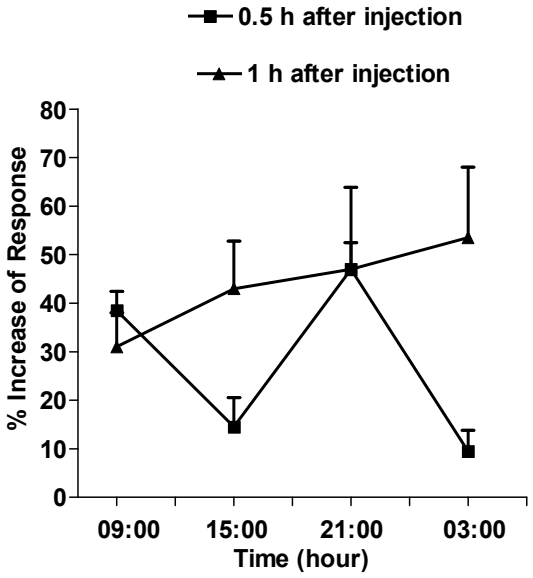

Figure.2. Circadian variation of antinociceptive effect of adenosine on mice in the hot-plate test. Each point represents the mean ± S.E.M. of response for *n=6* experiments on mice. Maximum antinociceptive activity was observed at 21:00, 0.5 h after injection ($P < 0.05$, Tukey–Kramer test).

03:00 of the day (p<0.05). In previous studies have been reported that the minimum sensitivity of pain in mice was observed in dark phase (19). Our results showed that adenosine, a neural modulator, have maximum antinociceptive effect at 21:00, therefore antinociceptive effect of this agent was time dependent. There is relevance between times after injection, different times of day and antinociceptive activity of adenosine.

The circadian variation of adenosine and its metabolisms as well as metabolizing enzymes and even the specific receptors has been demonstrated in previous studies (13). Adenosine showed a low half-time and rapid metabolism (7), also it was shown that

adenosine-metabolizing enzymes are more active in light phase. Therefore the peak of its antinociceptive effect was observed in the dark phase (21:00 PM). Due to the low half-time of adenosine, its rapid metabolism and ineffectiveness of i.p. administration of low dose, in this study high dose of adenosine was used and also two A1 agonists of adenosine, 2-CLAdo and R-PIA, were selected. The results

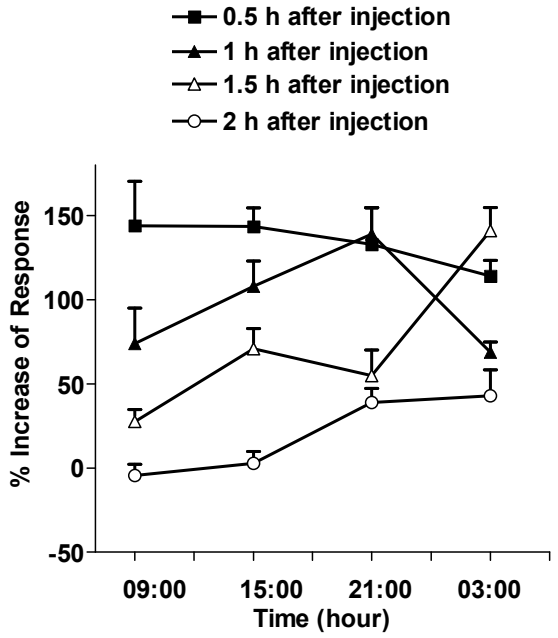

Figure.3. Circadian variation of antinociceptive effect of 2-CLAdo on mice in the hot-plate test. Each point represents the mean ± S.E.M. of response for *n=6* experiments on mice. Maximum activity was observed in dark phase in 1, 1.5 and 2 h after injection ($P < 0.05$, Tukey–Kramer test).

of 2-CLAdo indicated that the maximum of antinociceptive effect was in dark phase and results of R-PIA exhibited that peak of antinociceptive activity was in light phase. The difference between time dependency of 2-CLAdo and R-PIA may be related to different circadian variation of metabolism and elimination systems of these agents. As A1 receptor has a circadian rhythm in nervous system (20, 21) and 2-CLAdo, as well as R-PIA is A1 receptor agonists, there is time dependency for them and our results confirmed it. Also there is relevance between times after injection, different times of day and antinociceptive activity of 2-CLAdo and R-PIA. In this study the antinociceptive effect of adenosine was observed in the hot plate test that is used for evaluation of central antinociceptive effect, because adenosine cannot pass blood-brain barrier (15), it is probably related to secondary effects on neurotransmitter release. On the other hand, pain is a complex phenomenon and intensity of pain may be varried by many factors. So contrast findings

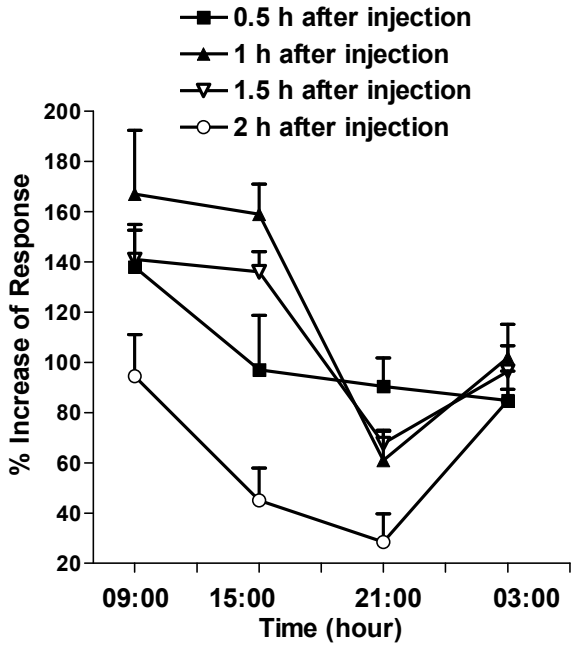

Figure 4. Circadian variation of antinociceptive effect of R-PIA on mice in the hot-plate test. Each point represents the mean ± S.E.M. of response for *n=6* experiments on mice. Maximum activity was observed in light phase in 1, 1.5 and 2 h after injection ($P < 0.05$, Tukey–Kramer test).

that have been observed in different studies may be related to methodological differences. Further research should be aimed at characterizing the chronobiology of pain in different experimental and clinical conditions.

The results of this study indicated that the pattern of pain in male mice have a circadian variation. The antinociceptive effects of adenosine and its two analogues were time dependent. These circadian variations in antinociceptive activity may be important in the administration of these agents.

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