

Effect of Circadian Rhythm on Antiulcer Activity of Hydroxyzine in Cold Stress Induced Ulcer in Wistar Albino Rats

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ABSTRACT

Circadian rhythms are driven by endogenous clock gene and affects in several gastric parameters causing ulcerogenesis. The study was aimed to evaluate the time dependent antiulcer activity of hydroxyzine in cold-stress-induced ulcer model. The ulcer protection effect of hydroxyzine was studied at various time intervals (9 am, 1 pm, 5 pm, 9 pm, 1 am and 5 am) in cold stress ulcer model using wistar albino rats. Comparison of hydroxyzine and control group showed significant ($p < 0.05$) decrease in gastric acidity at 9 pm. The free acidity was reduced significantly ($p < 0.001$) at 5 am within 24 h time. No significant change was observed in free acidity in control and hydroxyzine-treated animals. The total acidity was significantly ($p < 0.001$) reduced in hydroxyzine group at 5 am within 24 h time. Comparison between hydroxyzine and control group at 1 am showed significant ($p < 0.01$) reduction in total acidity in hydroxyzine treated animals. Ulcer index was found to be significantly low ($p < 0.001$) in hydroxyzine-treated animals at 5 am within 24 h. The ulcer index of hydroxyzine-treated animals was significantly ($p < 0.001$) reduced at 9 am as compared with control group of 9 am. Lipid peroxidation was significantly ($p < 0.01$) reduced in hydroxyzine-treated animals at 5 am within 24 h time. Mucin was significantly ($p < 0.001$) increased in hydroxyzine-treated animals at 1 am within 24-h time. The mucin content was significantly ($p < 0.001$) rose in hydroxyzine group at 5 am when compared to control at 5 am. The study reveals the role of biological clock on antiulcer activity of hydroxyzine.

Keywords: *Circadian rhythm, Antiulcer activity, Hydroxyzine, Antianxiety activity*

Chronopharmacology is the study of the manner and extent to which the kinetics and dynamics of medications are directly affected by endogenous biological rhythms, and also how the dosing time of medications affect biological timekeeping and the features (period, level, amplitude, and phase) of biological rhythms. Circadian rhythms effect on gastrointestinal pH can affect drug dissolution. Also, circadian rhythms effect on gastric emptying, motility, and blood flow can affect the rate, and in certain cases, the amount of drug absorption [1-2]. Studies showed that the time of drug administration, especially with reference to biological or circadian rhythms, can impact the kinetics and dynamics of various classes of

medications [3]. Chronokinetics refers to dosing-time, i.e., rhythm-dependent, differences in the absorption, distribution, metabolism, and elimination of medications [4,5]. Circadian rhythm in the physiology of gastrointestinal, such as gastric acid secretion is time dependent of light and dark phases [6,7]. Due to the rhythmic changes in its functional status, the susceptibility of the gastric acid secretion to noxious condition, including stress and chemical agent [5-8], have been shown to display time-dependent variation [9].

Hydroxyzine is a first generation of antihistamine of the diphenylmethane and piperazine used for the treatment of itching, allergies, anxiety, etc [10].

Anticholinergic action results from the direct interaction of this molecule with M₃ muscarinic receptors [9-11]. Stress ulcers are generally based on probable pathogenic mechanism like activation of parasympathetic nervous system which in turn leads to damage of gastric mucosa and further cause for ulceration [12]. Drugs used in treatment of anxiety [13] had reported anti-secretory and antiulcer activity and thus recommended in treatment of gastric ulcer alone or in combination [9,10]. This study was undertaken with an objective to evaluate the effect of circadian rhythm on antiulcer activity of hydroxyzine in cold stress induced ulcer.

MATERIALS AND METHODS

Drugs and chemicals

Hydroxyzine HCl injection U.S.P. (Atarax®) manufactured by UCB India Pvt. Ltd. was purchased. Alcian blue 8 GX was purchased from LOBA Chemical (Pune, India). All other reagents and chemicals were purchased analytical grade with high purity.

Experimental animals

Local bred Wistar albino rats of both sexes weighing 150-200 g were used in this study. Animals were synchronized for circadian rhythm study by maintaining them under controlled environmental conditions (temperature, feeding time, light and dark period, etc.). The lighting regimen was 12 h of light and 12 h of darkness with a light intensity of about 100 lux [9]. Lighting was provided with cool fluorescent bulbs. Photo safe red bulbs were used to visualization during dark. Animals were provided food and water ad libitum during the synchronization period and deprived of food expect water for 24 h before the ulcerogenic stimuli. Experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Smt. Kashibai Navale College of Pharmacy, Pune. (No.SKNCOP/IAEC/18/2011-12).

Experimental protocol

Cold restraint stress-induced ulcers

A modified procedure was used for induction of stress-induced gastric mucosal damage. After fasting for 24 h, the animals (n=6) were immobilized by means of a wire net firmly fitted to the body of the rats, and the rats were placed in a cold temperature at 2°C for 2 h [14]. The immobilization procedure began at 7 am, 11 am, 3 pm, 7 pm, 11 pm and 3 am for control and hydroxyzinetreated animals. At the end of the 2 h stress, the animals were sacrificed following light ether anaesthesia. The stomach was removed, opened along the greater curvature, rinsed with saline solution, and scored macroscopically for mucosal damage [15]. In hydroxyzine-treated animals, intraperitoneal injection of hydroxyzine was given as a single dose of 32 mg/kg [16] to each animal. The same aforementioned clock time was used in the restraint-cold stress application and

2 h after the dose of hydroxyzine and immobilization period (stress).

Control group

Group I: (9:00 am), Group II: (1:00 pm), Group III: (5:00 pm), Group IV: (9:00 pm), Group V: (1:00 am) and Group VI: (5:00 am) was treated with sterile water for injection (SWFI) 1 ml i.p. Immediately after administration of SWFI, all the animals were restrained and subjected for cold stress for 2 h.

Hydroxyzine group

Group I: (9:00 am), Group II: (1:00 pm), Group III: (5:00 pm), Group IV: (9:00 pm), Group V: (1:00 am) and Group VI: (5:00 am) was treated with hydroxyzine 32 mg/kg i.p. Immediately after administration of hydroxyzine, animals were restrained and subjected for cold stress for 2 h. Animals from the control and treated groups were sacrificed after 2 h immobilization period for estimation of different parameters like gastric volume, pH, free acidity, total acidity, Na⁺, K⁺, lipid peroxidation, mucin and ulcer index.

Estimation of parameters

Collection of Gastric Juice

The stomach was excised carefully by keeping the oesophagus closed and opened along the greater curvature and the luminal contents were removed. The gastric contents were collected and centrifuged at 1000 rpm for 10 min; the volume of the supernatant was expressed as ml/100 gm body weight and the centrifuged samples were decanted and analysed for gastric volume, pH, free acidity and total acidity [17]. The stomach was washed with saline and observed for gastric lesion using a dissecting microscope. Ulcers were scored and the ulcer index was determined [16-18].

Estimation of pH

About 1 ml of supernatant liquid was pipetted out and diluted to 10 ml with distilled water. pH of this solution was noted with use of pH meter [17].

Estimation of free and total acidity

About 1 ml of gastric juice was pipetted into a 100-ml conical flask, two or three drops of Topfer's reagent were added and this was titrated with 0.01 N sodium hydroxide [19] until all traces of red colour disappeared and the colour of the solution became yellowish-orange. The volume of alkali added was noted. This volume corresponds to free acidity [20]. Two or three drops of phenolphthalein solution were added and titration was continued until a definite red tinge appeared. The total volume of alkali added was noted. The volume corresponds to the total acidity [21,22].

Estimation of Sodium and Potassium concentration in gastric juice

Sodium stock solution was prepared by dissolving 2.542 g sodium chloride in 11 mL of distilled water. It

Table 1. Effect of hydroxyzine hydrochloride on volume of gastric juice (mL)

	9 am	1 pm	5 pm	9 pm	1 am	5 am
Control	2.00 ± 0.24	1.88 ± 0.28	3.05 ± 0.25	4.48 ± 0.22	4.81 ± 0.15	3.56 ± 0.24
Hydroxyzine (32 mg/kg)	1.23 ± 0.13	1.18 ± 0.13 ^{@@@}	2.31 ± 0.30	3.31 ± 0.11	3.18 ± 0.26	1.60 ± 0.31

Values are mean ± SEM; n=6 in each group; ^{@@@}*p* < 0.001 compared with hydroxyzine group within 24h (ANOVA followed by Bonferroni test)

Table 2. Effect of hydroxyzine hydrochloride on pH

	9 am	1 pm	5 pm	9 pm	1 am	5 am
Control	2.04 ± 0.33	1.79 ± 0.18	2.32 ± 0.48	1.48 ± 0.14	0.28 ± 0.14	1.79 ± 0.14
Hydroxyzine (32 mg/kg)	1.26 ± 0.16	1.49 ± 0.14	1.47 ± 0.17	1.15 ± 0.07 [*]	1.26 ± 0.16	1.33 ± 0.08

Values are mean ± SEM; n=6 in each group; ^{*}*p* < 0.05 compared with control group at 9 pm (ANOVA with Student's *t* test)

Table 3. Effect of hydroxyzine hydrochloride on free acidity (mEq/L/100 g)

	9 am	1 pm	5 pm	9 pm	1 am	5 am
Control	42.67 ± 1.92	50.83 ± 1.55	37.00 ± 1.23	53.67 ± 1.5	56.50 ± 1.31	19.50 ± 2.52
Hydroxyzine (32 mg/kg)	30.17 ± 2.54	35.33 ± 1.96	24.83 ± 1.81	43.33 ± 2.04	24.00 ± 2.26	13.00 ± 1.21 ^{@@@}

Values are mean ± SEM; n=6 in each group; ^{@@@}*p* < 0.001 compared with hydroxyzine group within 24 h (ANOVA followed by Bonferroni test)

contains 1 mg sodium per mL (i.e. 1000 ppm). Stock solution was diluted to give four solutions containing 10, 5, 2.5 and 1 ppm of sodium ions.

The potassium stock solution was prepared by dissolving 1.099 g potassium chloride in 11 mL of distilled water [23]. It contains 1 mg potassium per mL (i.e. 1000ppm). Stock solution was diluted to give four solutions containing 20, 10, 5 and 2 ppm of potassium ions. The sodium and potassium in the gastric juice was determined using a Systronics Mediflame-152 flame photometer. For sodium and potassium, the flame intensity corresponding to the concentrated of stock solution was noted by using appropriate filters. The concentration of the sodium and potassium ions was calculated from the graph and expressed in terms of meq/L [23].

Estimation of ulcer index

The inner surface of free stomach was examined for gastric lesions. The number of ulcers was counted [24,25].

Estimation of lipid peroxidation (LPO)

Lipid peroxidation (TBARS method) was measured using MDA equivalents derived from tetraethoxypropane. MDA and other aldehyde have been identified as products of LPO that reacts with thiobarbituric acid to give pink coloured species that absorbs at 532 nm. The method involved heating of biological sample with TBA reagent for 20 min in a boiling water bath. TBA reagent contains 20% trichloroacetic acid, 0.5% thiobarbituric acid and 2.5% N HCl. After cooling, the solution was centrifuged at 2000 rpm for 10 min and precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm against the blank [26,27].

Estimation of Mucin

After the collection of gastric juice, the glandular portion excision that opened the lesser curvature was opened. The everted stomachs were soaked for 2 h in 0.1% alcian blue 8GX dissolved in 0.16 M sucrose buffered with 0.05 M sodium acetate adjusted to a pH with hydrochloric acid. Uncomplexed dye was removed by two successive washes of 15 and 45 min in 0.25 M sucrose solution. Dye complex with mucus was diluted by immersion in 10 ml aliquots of 0.5 M magnesium chloride for 2 h. The resulting blue solutions were shaken briefly with an equal volume of diethyl ether and the optical density of the aqueous phase was measured at 605 nm using a Jasco spectrophotometer. The mucin content of the sample was determined, which has been expressed in microgram/gram of wet gland tissue [21].

Statistical analysis

The results are presented as mean ± SEM. The significance of circadian rhythmicity in each testing procedure was statistically analysed using analysis of variance (ANOVA) followed by Bonferroni multiple comparison test and differences between groups at the same stage were tested with unpaired Student *t* test.

RESULTS

Effect of hydroxyzine hydrochloride on volume of gastric juice

Hydroxyzine decreases the gastric juice secretion significantly (*p*<0.001) at 1 pm within 24 h time. When comparison was made between hydroxyzine and control animals, no significant change was observed in the gastric juice level as presented in Table 1.

Table 4. Effect of hydroxyzine hydrochloride on total acidity (mEq/L/100g)

	9 am	1 pm	5 pm	9 pm	1 am	5 am
Control	68.67 ± 1.30	79.67 ± 1.08	57.67 ± 4.07	84.17 ± 1.64	88.83 ± 0.79	25.33 ± 1.85
Hydroxyzine (32 mg/kg)	51.33 ± 1.49	62.67 ± 1.56	39.50 ± 2.59	69.50 ± 2.12	34.17 ± 3.27**	22.17 ± 1.95 ^{@@@}

Values are mean ± SEM; n=6 in each group; ** $p < 0.01$ compared with control group at 1 am (ANOVA with Student's t test), ^{@@@} $p < 0.001$ compared with hydroxyzine group within 24 h (ANOVA followed by Boneferronis test)

Table 5. Level of Na⁺ (mEq/L) gastric juice of animals treated with hydroxyzine

	9 am	1 pm	5 pm	9 pm	1 am	5 am
Control	11.37 ± 0.37	12.85 ± 0.34	12.82 ± 0.38	28.23 ± 0.93	27.50 ± 0.70	2.93 ± 0.21
Hydroxyzine (32 mg/kg)	9.58 ± 0.40	10.43 ± 0.31	10.42 ± 0.46	17.75 ± 1.25	12.63 ± 0.78	3.68 ± 0.38 ^{@@@}

Values are mean ± SEM; n=6 in each group; ^{@@@} $p < 0.001$ compared with hydroxyzine group within 24 h (ANOVA followed by Boneferronis test)

Table 6. Level of K⁺ (mEq/L) gastric juice of animals treated with hydroxyzine

	9 am	1 pm	5 pm	9 pm	1 am	5 am
Control	6.28 ± 0.43	4.80 ± 0.22	5.03 ± 0.40	5.98 ± 0.27	5.31 ± 0.64	1.39 ± 0.20
Hydroxyzine (32 mg/kg)	5.66 ± 0.45 ^{@@@}	4.26 ± 0.30	3.98 ± 0.24	4.86 ± 0.41	2.21 ± 0.46	1.38 ± 0.2

Values are mean ± SEM; n=6 in each group; ^{@@@} $p < 0.001$ compared with hydroxyzine group within 24 h (ANOVA followed by Boneferronis test)

Effect of hydroxyzine hydrochloride on pH

The pH of gastric juice was not reduced significantly in hydroxyzine group within 24 h time. Comparison of hydroxyzine and control group showed significant ($p < 0.05$) decrease in gastric acidity at 9 pm as presented in Table 2.

Effect of hydroxyzine hydrochloride on free acidity

The free acidity was reduced significantly ($p < 0.001$) at 5 am within 24 h time. No significant change was observed in free acidity in control and hydroxyzine-treated animals as presented in Table 3.

Effect of hydroxyzine hydrochloride on total acidity

The total acidity was significantly ($p < 0.001$) reduced in hydroxyzine group at 5 am within 24 h time. Comparison between hydroxyzine and control group at 1 am showed significant ($p < 0.01$) reduction in total acidity in hydroxyzine treated animals as presented in Table 4.

Ulcer index of animals treated with hydroxyzine hydrochloride

Ulcer index was found to be significantly low ($p < 0.001$) in hydroxyzine-treated animals at 5 am within 24 h time. The ulcer index of hydroxyzine-treated animals was significantly ($p < 0.001$) reduced at 9 am compared with control group of 9 am. Significant decrease ($p < 0.01$), ($p < 0.01$) and ($p < 0.01$) in ulcer index was observed in hydroxyzine-treated animals at 1 pm, 9

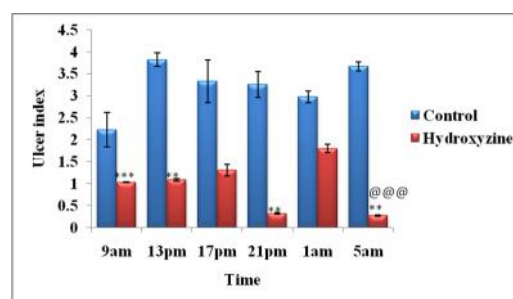
pm and 5 am when compared with control at same timing (Fig 1).

Level of Na⁺ in gastric juice of animals treated with hydroxyzine

The Na⁺ concentration was reduced very significantly ($p < 0.001$) at 5 am within 24 h time. No significant change was observed in the level of Na⁺ in hydroxyzine and control animals presented in Table 5.

Level of K⁺ in gastric juice of animals treated with hydroxyzine

K⁺ concentration was significantly ($p < 0.001$) increased at 9 am in hydroxyzine-treated animals within 24 h time. No significant change was observed when

**Fig 1.** Ulcer index of animals treated with hydroxyzine hydrochloride

Values are mean ± SEM; n=6 in each group; ** $p < 0.01$ compared with control group at 13 pm, 21 pm and 5 am (Student's t test), *** $p < 0.001$ compared with control group at 9 am (ANOVA with Student's t test), ^{@@@} $p < 0.001$ compared with hydroxyzine group within 24 h (ANOVA followed by Boneferronis test)

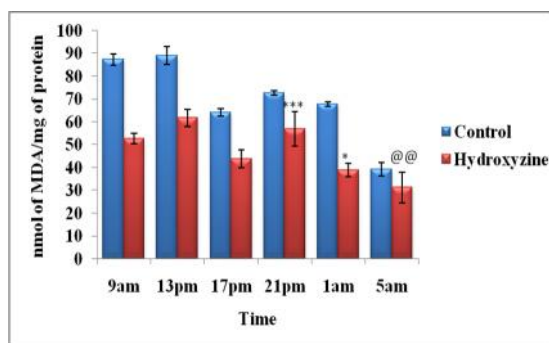


Fig 2. Effect of hydroxyzine hydrochloride on Lipid peroxidation (nmol of MDA/ mg of protein) in rat stomach

Values are mean \pm SEM; n=6 in each group; * $p < 0.05$ compared with control group at 1am (ANOVA with Student's t test), *** $p < 0.001$ compared with control group at 21 pm (ANOVA with Student's t test), @ $p < 0.01$ compared with hydroxyzine group within 24 h (ANOVA followed by Bonferroni's test)

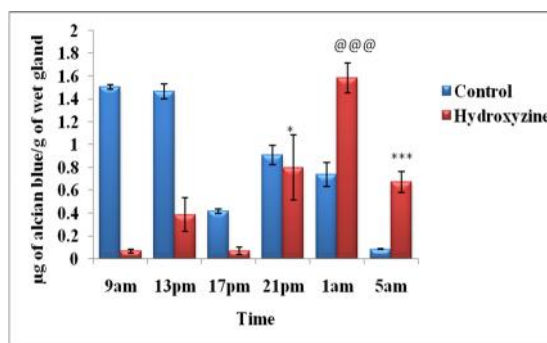


Fig 3. Effect of hydroxyzine hydrochloride on mucin (μ g of alcian blue/g of wet gland) level

Values are mean \pm SEM; n=6 in each group; * $p < 0.05$ compared with control group at 21 pm (ANOVA with Student's t test), *** $p < 0.001$ compared with control group at 5 am (ANOVA with Student's t test), @ $p < 0.01$ compared with hydroxyzine group within 24 h (ANOVA followed by Bonferroni's test)

control was compared with hydroxyzine-treated animals as presented in Table 6.

Effect of hydroxyzine hydrochloride on lipid peroxidation of rat's stomach

Lipid peroxidation was significantly ($p < 0.01$) reduced in hydroxyzine-treated animals at 5 am within 24 h time. Significant reduction ($p < 0.001$) and ($p < 0.05$) in lipid peroxidation was observed in hydroxyzine-treated animals at 9 pm and 1 am when compared with control group at 9 pm., and 1 am as presented in Fig 2.

Effect of hydroxyzine hydrochloride on mucin level

Mucin was significantly ($p < 0.001$) increased in hydroxyzine-treated animals at 1 am within 24 h time. The mucin level was significantly ($p < 0.001$) raised in hydroxyzine group at 5 am when compared to control at 5 am as presented in Fig 3.

DISCUSSION

Chronopharmacology studies time-dependant effect of drugs. An alteration in drugs pharmacodynamics and kinetics takes place due to variation in biological rhythm [28]. There are several factors that may induce ulcer in human beings such as: stress, chronic use of anti-inflammatory drugs and continuous alcohol ingestion [10]. Several studies revealed time-dependent variation in gastric mucosa due to ulcerogenic stimuli consisting of stress [15].

The possible mechanism leading to the development of acute to stressful conditions is the central as well as peripheral activation of cholinergic system [29,30], thereby increased secretion of vagal nerve and release of acetylcholine which binds to M_3 receptor in the rat stomach is crucial [31,32].

Circadian fluctuation as a central functions in the brain, along with circadian rhythm in local gastric defense mechanism; seemingly contribute to the

observed time-dependent susceptibility of rat gastric mucosa to restraint cold injury [33]. In the restraint cold model, administration of hydroxyzine 32 mg/kg i. p showed decreased gastric volume at 1 pm which indicates the maximum antisecretory activity of hydroxyzine at this time. Significant decrease in pH at 9 pm indicates its maximum effectiveness over decreasing pH at this time. Lower values for total acidity and ulcer index were observed at 5 am representing a potential anti-ulcer activity at this time. Also the drug showed similar effect at 1 pm and 9 pm. The gastric secretion is regulated by the hydroxyzine by blocking M_3 receptor and this may be the basis for producing the anti-ulcer and anti-secretory action. Also the drug hydroxyzine was found to decrease the lipid peroxidation at 5 am, 9 pm and 1 am indicating antioxidant-like activity. Thus, the gastric cellular damage was prevented by the drug by increasing the secretion mucin at 1 am and 5 am. The results obtained from present study support the anticholinergic, vagolytic and anti-secretory [33] action of hydroxyzine [8]. Prominent ulcer protective action was observed at 5 am. The study undertaken explores the effect of circadian rhythm on antiulcer activity of hydroxyzine.

In conclusion, circadian rhythm is present for the body temperature, heart rate, blood pressure, organ blood flow, pulmonary and kidney function, as well as for concentration of neurotransmitters, hormones, enzymes, electrolytes and glucose. Action of drugs used in treatment of diseases is affected by the circadian rhythm. There is alteration in the pharmacodynamic and pharmacokinetic activity of the drug in 24 h time. Also, there is change in the behavior in the expression of enzymes and proteins which interacts with the drugs. Present study reveals the anti-ulcer activity of drug hydroxyzine which get varied as per the time within 24 h. The drug had showed significant antiulcer activity at night time as compared to day time. Our study clearly reflect that in rodents hydroxyzine effect i.e. either anti-anxiety, anti-secretory and/or antiulcer varies as per

the time and this may form the basis for deciding the right time of drug administration in humans after further study.

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