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Protective Effect of β-Carotene Extracted From the Cyanobacterium *Oscillatoria brevis* Against Stress-Induced Alterations of Circadian Behavior and Oxidative Markers rhythms in Rat

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***ABSTRACT*:** *Health problems related to stress is a major global issue. The present study aims at evaluating the possible protective effect of β-carotene (βC), as a natural cyanobacterial product, against stress-induced alterations in circadian rhythms of behavioral activities and oxidative markers. Male albino rats were subjected to chronic unpredictable stress (CUS) for 21 days. Rats were randomly divided into 4 groups (20 rats/group) viz.; control, CUS-exposed, βC-treated and βC-treated + CUS-exposed groups. One hour before testing, βC extracted from Oscillatoria brevis was administered (10 mg/kg) intraperitoneally (I.P.). Elevated plus maze (EPM) and forced swimming test (FST) were applied for behavioral assessment. Blood samples were collected at four circadian times (CT 3, 9, 15 and 21); 5 rats/time point, to monitor circadian profiles of nitric oxide (NO), lipid peroxidation (LPO) and glutathione (GSH). Circadian patterns were observed in all tested behavioral and antioxidant parameters. CUS induced anxiety- and depression-like behavior in rats where the immobility time in FST was significantly increased ($P<0.05$), whereas the time spent in open arms of EPM was decreased. Data showed that under CUS exposure the peak times (acrophase) of circadian rhythms of NO, LPO and GSH levels as well as immobility time and time spent in open arms were changed. βC improved these changes. CUS significantly increased NO, LPO, while it significantly decreased GSH levels ($P<0.05$). On the other hand, βC significantly decreased NO, LPO and immobilization time, meanwhile a significant increase of GSH and time spent in open arm was noticed ($P<0.05$). In conclusion, it can be suggested that βC has an antioxidant and neuroprotective influence in ameliorating both the anti-anxiety and anti-depression-like behavior as well as NO, LPO and GSH levels, in addition to modulating the altered circadian rhythms.*

***KEYWORDS**: β-carotene, circadian rhythm, CUS, Oscillatoria brevis, rat, stress*

I. INTRODUCTION

Stress may be an environmental, physical and/or psychological stimulus that is capable of altering the physiological homeostasis of the body, and hence it is considered as a crucial determinant in health and disease [1]. Circadian rhythms (CRs) are endogenous, about 24-hr oscillations controlling different physiological and behavioral functions of organisms from bacteria to humans. Stress responses and circadian rhythmicity are adaptations to environmental influences. Whereas stress responses are adaptations to unpredictable aspects of the environment, circadian rhythmicity is an adaptation to its predictable aspects, such as light-dark cycles [2]. The hypothalamic-pituitary-adrenal (HPA) axis and the extra-hypothalamic brain stress system mediate behavioral and neuroendocrine reactions to stress in animals and humans [3]. CRs of NO, LPO, and GSH was documented by Mastronardi *et al*. [4] and Subash and Subramanian [5]. On the other hand, several studies have demonstrated the effects of the circadian phase on multiple measures of depressive/anxiety-like behavior and (HPA) reactivity in a variety of mammals and primates [6,7]. Much attention has been focused on the protective biochemical function of naturally present antioxidants in biological systems, and on the mechanisms of their action [8]. Cyanobacteria are considered as good candidates for applications for example, in pharmaceuticals [9]. Carotenoids; as antioxidant, are the most widely distributed and structurally diverse classes of natural pigments predominantly produced by cyanobacteria [10]. In addition, *Oscillatoria sp*. contains high concentration pattern of β-carotene, as reported by [11]. The present study aims to clarify the protective effect of β-carotene, as a natural cyanobacterial product, against CUS induced alterations in rat's circadian behavior and oxidative markers which may play a crucial role in the treatment of a variety of diseases.

MATERIALS AND METHODS

Experimental animals: Eighty male albino rats weighing 140-180 g were used for the experimental procedures. Rats were obtained from the breeding unit of animal house in Zoology Department, Faculty of Science, Suez Canal University, Egypt, under (temperature 26ºC, natural light/dark cycles 14:12) conditions and allowed free access to food and water. They were maintained for one week in the experimental room habituation.

Sample collection and extraction of □-carotene: *Oscillatoria brevis* was obtained from the culture collection of algae, National Research Center, Dokki, Giza, Egypt. It was maintained in 20 ml of sterilized BG-11 medium. Three-week old stock cultures were transferred to 250-2000 ml fresh, sterilized medium at room temperature 25°C and supplied by sterilizing air [12]. *O. brevis* biomass was harvested from culturing medium and centrifuged (4500 rpm for 15 min) then biomass was dried in oven at 50°C. βC was extracted and isolated according to Pavia *et al.* [13]. Extracted βC was freshly prepared in a vehicle solution, consisted of 0.2% tween-80 in 0.9% NaCl solution. I.P. administration of βC extract (10 mg/kg b.wt.) was always applied one hour before the induction of CUS [14].

Experimental design: After one week of acclimation, rats were randomly divided into 4 equal groups (20 rats/group) viz. Control+vehicle (C): injected with vehicle, CUS+vehicle (S): injected with vehicle before CUS-exposure, β-carotene (β): injected with βC and CUS+βC (Sβ): injected with βC before CUS-exposure. To analyze the possible circadian phase role on depression-, anxiety-like behavior and oxidative markers data were sampled at 6-hour intervals over the 24-hour circadian time (viz. CT 3, 9, 15 and 21), 5 rats/time point. Sampling in the dark phase was conducted under red dim light to avoid any possible disturbance during the experiment.

Chronic unpredictable stress (CUS) Exposure: CUS is a widely used animal model for stress induction [15]. Rats were randomly received two different stressors every day along 21 days. The variety of stressors include cage rotation for 60 min, forced swimming in cold water at 10°C for 5 min, water and food deprivation for 16 hrs, electric shock for 5 min (electric current 1.0 mA conducted for 10 s, followed by 10 s rest, 30 times), 4-hrs wet bedding, 17-hrs isolation housing overnight and forced swimming in 25°C warm water. To exclude the effects of handling of the stressed rats, unstressed rats were also handled twice daily. C and β groups were housed in a separate room for three weeks and were not exposed to any kind of stress except handling for daily injection. All animal procedures and the experimental protocols were followed as approved by the research and ethics committee of Faulty of Science, Suez Canal University. Figure (1) illustrates the schedule of experiment protocol.

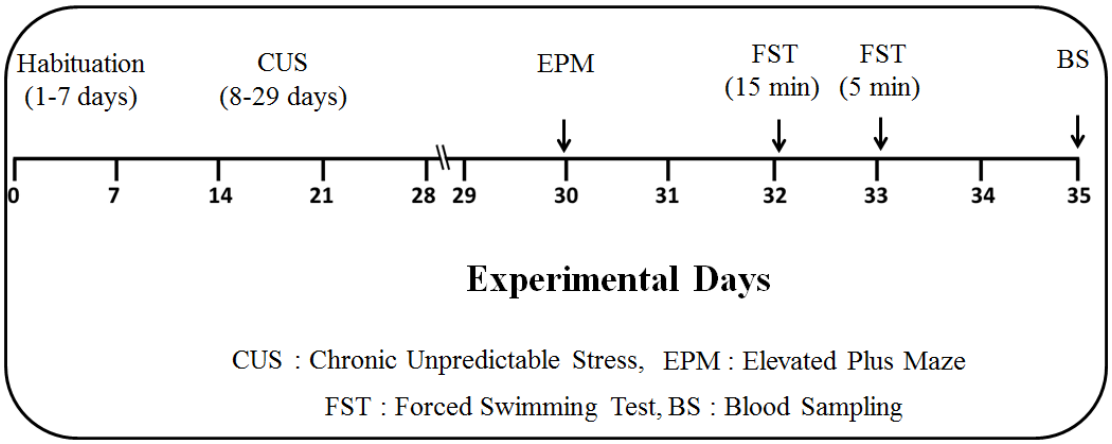

Figure (1). The schedule of the experimental protocol**.**

Elevated Plus Maze (EPM): According to Walf and Frye [16], anxiety levels were measured using the EPM test. The EPM is consists of two open arms (50 x 10 cm, each), two closed arms (50 x 10 x 30 cm, each) and a central platform (10 x 10 cm) and arranged in a way that the two arms of each type were opposite each other. The maze was elevated 50 cm above the floor. Each rat was individually placed in the EPM. At the beginning of each trial; animals were placed at the center of the maze facing an open arm. During a 5-min test period, anxiety-like behavior was quantified as the ratio between the time spent in the open arms and the total time spent in both open and closed arms. Entry into an arm was defined as the animal placing all four limbs onto the arm. The maze was thoroughly wiped clean with 5% ethanol solution after each trial.

Forced Swimming Test (FST): In order to assess the antidepressant activity of *O. brevis* extracts, the modified FST [17] was conducted. In the first trial, the rats were forced to swim in a glass cylinder (20 cm diameter, 50 cm in height containing 30 cm height freshwater at 23±1°C). The session was conducted through an initial 15-min training session (pre-test session) then, 24 hours later, a 5-min test session. During the test session, the immobility, swimming and climbing times were observed by video recording. The total duration of immobility was measured during the 5-min test. Upon removal from the water, rats were towel dried and finally returned to their cages.

Blood sampling and oxidative markers analysis: Blood was sampled 24 hours after the behavioral experiments and collected in EDTA test tubes. For GSH, the first part of blood samples were immediately evaluated [18] and for LPO and NO assessment, the second part of blood samples was centrifuged at 4000 rpm for 15-min and frozen at -20°C until next assay using (Bio-diagnostic, Giza, Egypt).

Statistical analysis: The values of variables were plotted as mean±SE. The mean values of animal groups were compared using one-way ANOVA followed by Post hoc Tukey test (IBM SPSS statistic 21 software) for multiple comparisons at each time. Differences were considered significant at $p<0.05$ level.

III. RESULTS

Under natural LD conditions and at room temperature (25ºC) all rats exhibited pronounced circadian patterns of behavioral activities and oxidative markers.

BEHAVIORAL ANALYSIS:

Elevated Plus Maze Test: Increase or decrease of anxiety-like circadian behavior were evaluated according to decrease or increase of time spent in open-arm respectively (Fig 2A). In unstressed rats, significantly ($p<0.05$) higher levels of anxiety-like behavior during light phase (9±1.5) was observed compared with dark phase (18.7±1.6). The acrophase of time spent in open-arm occurred at 21:00 h whereas its trough was at 09:00 h. On the other hand, CUS-exposure had significant effects and proved pronounced changes in anxiety-like circadian behavior pattern. Acrophases of rats exposed to CUS illustrated non-significant decrease ($p<0.05$) in time spent in open arms from (18.7±1.6) to (16.7±1.4) in S group when compared to C group, respectively. In S group, the peak of time spent in open arm was observed during the light phase at 15:00 h whereas its trough was at 03:00 h. At trough, non-significant decrease in time spent in open arms in S group from (9±1.5) to (5±1.2) when compared to C group. On the other hand, in β and Sβ groups, β-carotene extract administration exhibited no change in acrophase of the pattern of anxiety-like behavior when compared to C group (Fig. 2A). Furthermore, non-significantly increased time spent in open arms (17.9±2.4) was observed in Sβ group when compared with the S group (16.7±1.4) and its acrophase was illustrated at 21:00 h whereas its trough was at 09:00 h (Fig. 2A).

Forced Swimming Test: From the current results, depression-like behavior; evaluated as increased immobility time, was significantly oscillated in a circadian pattern (Fig 2B). The acrophase of depression-like behavior occurred at 03:00 h, whereas its trough was at 09:00 h in C group. After CUS-exposure, Tukey test proved a significant increase ($p<0.05$) in immobility time in S group from (151±9) to (161.2±13.4) when compared to C group, with an acrophase at 15:00 h instead of 03:00 h, respectively. At trough, non-significant increase was observed in immobility time in S group from (59±8.8) to (68±11) when compared to C group. No change was observed in the immobility time acrophase after β-carotene extract administration in β and Sβ groups, when compared to C group. Meanwhile, the amplitude profile revealed a significant alteration compared to C and S groups where a significant decrease ($p<0.05$) in immobility time was revealed (62.6±9) in β group when compared to C group (151±9) as shown in figure (2B). Also a significant decrease ($p<0.05$) in immobility time was elucidated (72.7±6.1) in Sβ group when compared to S group (161.2±13.4) and its acrophase, was displayed at 03:00 h (Fig. 2B).

Oxidative markers analysis: In the present study, the oxidative markers under investigation showed marked fluctuations over 24-h periods (Fig 3). Tukey test revealed detectable and significant alterations ($p<0.05$) expressing circadian patterns over the 24-h period inside each group. In C group, rats showed the acrophase of reduced GSH level at 09:00 h, as shown in figure (3A). Meanwhile, the acrophases of plasma NO and LPO levels were reached at 21:00 h (Fig. 3B & C). CUS-exposure revealed apparent changes in circadian parameters such as amplitude and acrophase of the rhythm. Under CUS, when comparing S group to C group, a significant decrease ($p<0.05$) in GSH level from (1023±18) to (602±1.4) was noticed and its acrophase was at 21:00 h (Fig. 3A) whereas its trough was at 09:00 h. During trough time, a significant decrease ($p<0.05$) was recorded in GSH from (898±53) to (381±69) when compared to C group. Levels of LPO and NO were significantly increased ($p<0.05$) in S group (24±0.5 and 691±12) when compared to C group (15±0.8 and 526±34, respectively). Acrophases of both LPO and NO circadian patterns in S group were reached at 09:00 h whereas its trough was

at 03:00 h and 21:00 h, respectively. (Fig. 3B & C). Also, a significant ($p<0.05$) increase was observed at trough of LPO and NO from (8±1.5 and 426±30) to (15±3 and 611±25, respectively) when compared to C group.

After β-carotene extract administration, the results showed no changes in acrophase of the circadian pattern, when comparing C with both β and Sβ groups. Acrophases were at 09:00 h for GSH whereas its trough was at 21:00 h but at 21:00 h for LPO, NO whereas their trough was at 09:00 h (Fig. 3). Tukey test confirmed the significant increase ($p<0.05$) in amplitude of GSH to 1188±11 and 1137±18 in β and Sβ groups in comparison with C and S groups, respectively. On the other hand, β-carotene significantly decreased (Tukey test, $p<0.05$) the level of LPO to 11±0.7 and 13±0.6 in β and Sβ groups, when compared to C and S groups, respectively. The amplitude of NO level was significantly decreased (Tukey test, $p<0.05$) to 480±13 and 589±43 in β and Sβ groups, when compared to C and S groups, respectively, as shown in figure (3).

IV. DISCUSSION

The present study provides evidence that the CUS-exposure induced alternations in the CRs of behavioral activities as well as oxidative stress markers, namely NO, LPO and GSH in rats. Furthermore, β-carotene, as a natural cyanobacterial product, indicated its protective role in ameliorating the disturbed parameters as well as modulating their CRs. In the current study circadian patterns of oxidative markers and behavioral activities were evidenced. The oxidative stress indicators; NO, LPO and GSH were monitored because they are useful to understand the oxidative damage cascades in depression and anxiety disorders [19]. Our results demonstrated that in unstressed rats the acrophases of plasma LPO and NO were reached during the subjective night (at 21:00 h) as previously reported by Mastronardi *et al*. [4]. Contrariwise, GSH peak was observed during the subjective day (at 9:00 h) in control group, which is comparable with that mentioned by Subash and Subramanian [5] where GSH peak was at 7:20 h. The EPM and FST are the most widely used animal models of anxiety and depression [20]. Anxiety- and depression-like behavior in the present study expressed circadian patterns. Depression-like behavior increased during subjective dark phase in unstressed rats as being reported in Kelliher *et al.* [21]. On the contrary, anxiety-like behavior increased during subjective light phase in unstressed rats, as mentioned by Bilu and Kronfeld-Schor [22].We adopted the CUS as a well-validated animal model of depression and anxiety [15]. Previous studies reported that chronic stress revealed CRs variations such as in sleep [23], locomotor activity [24], body temperature [25] and hormone secretion [26]. Supplementary, current study showed that under CUS conditions there was an increase in both NO and LPO levels. Their peak was recorded at 9:00 h expressing a phase shift of about 12 hours. This increment may be attributed to the CUS-exposure which results in the production of NO [27] and this initiates and propagates LPO production [28]. Under the current CUS conditions, lower GSH level profile accompanied by about 12-hour phase shift was recorded. Chen *et al*. [29] and Tishkina *et al*. [30] mentioned that chronic stress increases free radical oxidation processes in brain and other organs. In this study, after CUS-exposure, a significant increase in the immobility time in FST was observed, indicating a depression-like behavior [17], this result concord with Aslani *et al.* [31]. The CUS-induced depression-like behavior may be due to altered glucocorticoid levels which have a role in the development of depression [32].

Stress leads to activation of the hypothalamic-pituitary-adrenal (HPA) axis. There is growing evidence supporting the interactions between the circadian and stress systems. Anatomically, the circadian and stress signals converge at the paraventricular nucleus (PVN) in the hypothalamus. Therefore the daily rhythm of HPA activity is dependent on the suprachiasmatic nucleus (SCN) clock [33,34]. Genes that are involved in the operation of the circadian and stress systems are expressed in the PVN [35]. The *PER* genes (*PER1, PER2 and PER3*) in general are part of the central CR organization system in the SCN. Whereas the central circadian oscillator appears to be well-protected against unpredictable stressful stimuli, certain stressors can strongly affect the output of the clock and the expression of the rhythm [36]. It is documented that some stressors are found to influence the *Per1* mRNA expression [37,27]. Subsequently, the observed phase shifting in our results may be due to the increase in the NO levels where it is well known that NO is implicated in the regulation of CRs [38]. Furthermore, it is documented that a gene-stress interaction effect on the transcription of *Per1*, possibly results in different circadian organization under stressful conditions [39], in addition to the reversible changes of expression of the period clock gene (*PER2*) rhythms in the SCN caused by CUS, as being adopted by Jiang *et al*. [40]. β-carotene functions as a radical-trapping antioxidant at low oxygen pressure to reduce the extent of nuclear damage and to inhibit lipid peroxidation [41,42] and can increase GSH concentrations [43]. Therefore βC can scavenge free radicals directly by acting as an antioxidant. These data are in agreements with the present study where βC decreased both NO and LPO and increased GSH levels in both unstressed and stressed rats. On the other hand, the current results proved that βC decreased the immobilization time in FST and increased the opening spent time in EPM of both unstressed and stressed rats, indicating its anti-depressant and anti-anxiety modulation. This is in agreement with Dhingra and Bansal [44] who reported a similar

significant anti-depressant-like activity of βC in mice. This may be attributed to the inhibition of oxidative stress and scavenge of free radicals especially NO [45].

V. CONCLUSION

In conclusion stress responses and circadian rhythmicity are adaptations to environmental influences. The circadian patterns of oxidative markers and behavioral activities observed in rats were affected following the CUS-exposure conditions. However, βC can ameliorate CUS adverse effects as well as may have a modulating role on the induced circadian alternations.

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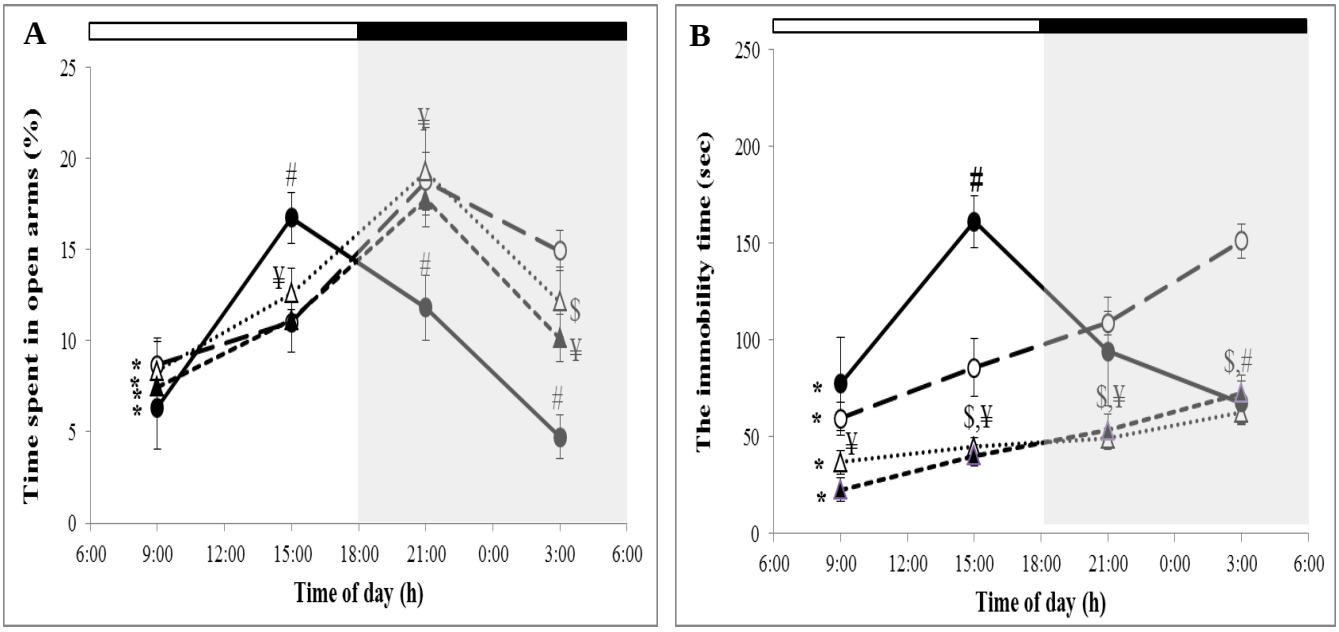

Figure 2. Effect of β-carotene extract administration on circadian patterns of **(A)** anxiety-like behavior evaluated by Elevated Plus Maze and **(B)** depression-like behavior evaluated by Forced-Swimming Test in stress-induced rats. Experimental groups are: C= control (open circle), β= having β-carotene extract (open triangle), S= stress-induced (closed circle) and Sβ= stress-induced+β-carotene extract (closed triangle). "White bars" and "dark bar with shaded area" refer to the natural day and night phases, respectively. Each value represents the mean±SE (20 rats/group; 5 rats/time point). Data was analyzed by one-way ANOVA test followed by Tukey post hoc test. Statistical significance is indicated at $p<0.05$ level as following:

*: comparison inside each group,
$: comparing β vs C groups.
#: comparing S vs C groups.
¥: comparing Sβ vs S groups.

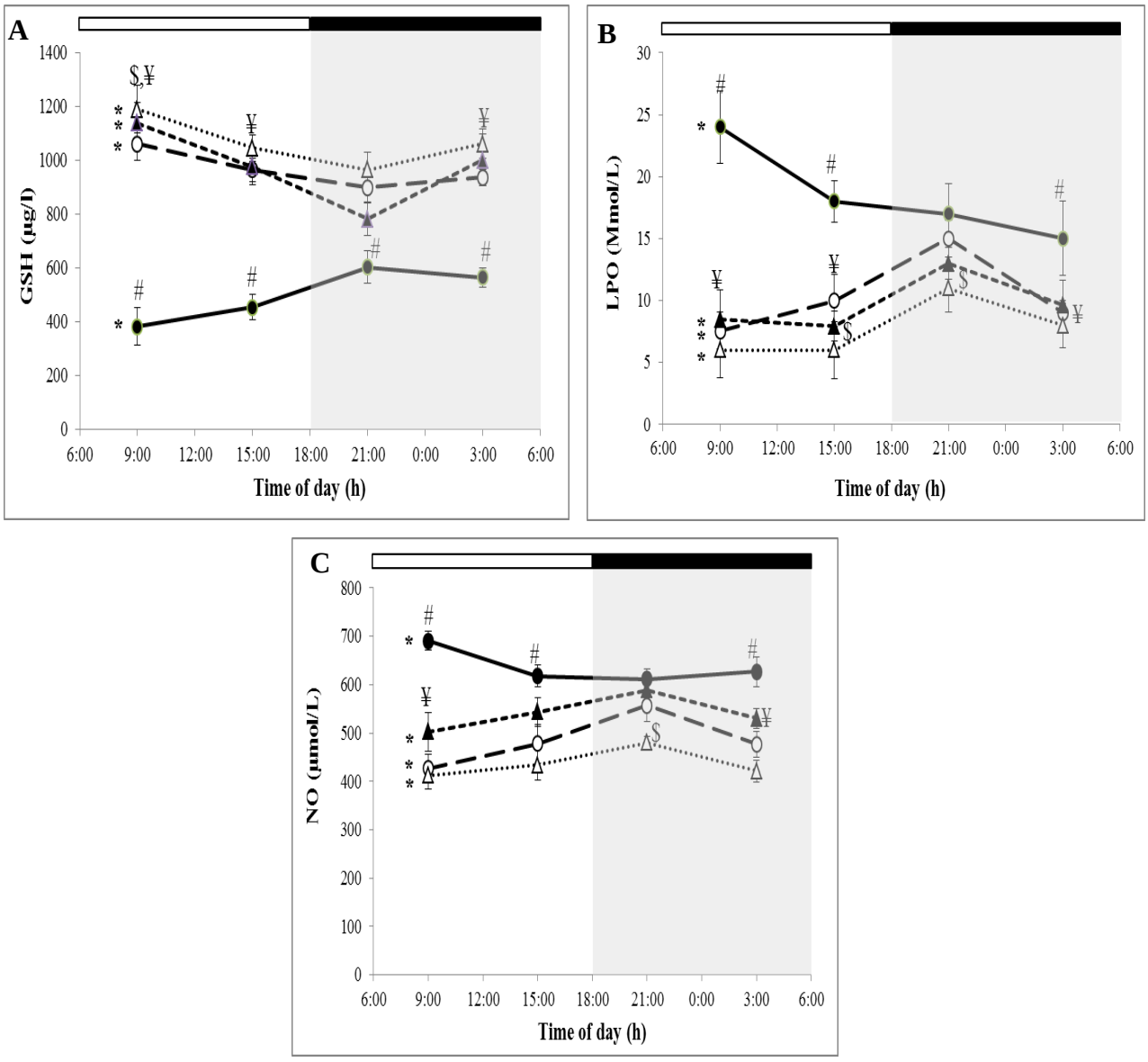

Figure 3. Effect of β-carotene extract administration on circadian patterns of **(A)** reduced glutathione (GSH), **(B)** Lipid peroxidation (LPO) and **(C)** Nitric oxide (NO) in stress-induced rats. Experimental groups are: C=control (open circle), β= having β-carotene extract (open triangle), S=stress-induced (closed circle) and Sβ= stress-induced+β-carotene extract (closed triangle). "White bars" and "dark bar with shaded area" refer to the natural day and night phases, respectively. Each value represents the mean±SE (20 rats/group; 5 rats/time point). Data was analyzed by one-way ANOVA test followed by Tukey post hoc test. Statistical significance is indicated at $p<0.05$ level as following:
*: comparison inside each group.
$: comparing β vs C groups.
#: comparing S vs C groups.
¥: comparing Sβ vs S groups.