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RESEARCH ARTICLE

Effect of Weightlessness on Neutrophils and Lymphocytes of Rats

Khusi Muhammad Saqib, Zia-ur-Rahman[1] and Saeed Ahmad Nagra[2]

[1]Department of Physiology and Pharmacology, University of Agriculture, Faisalabad; [2]Institute of Chemistry and Biochemistry, University of the Punjab, Lahore, Pakistan
*Corresponding author: drziar@yahoo.com

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ABSTRACT

In the present study, two hundreds and forty healthy albino young (n=120) and old (n=120) rats were used during winter and summer season. Rats were divided into four groups in each season i.e. young and old, consisting of male (n=30) and female (n=30) in each age category. In each age × sex matched rats, three subgroups were made and have been given the name as cage control (CC) group, horizontal restrained group (HR) and head down suspended (HDS) group. For winter season, the room temperature of experimental period ranged from 20 to 23°C and for summer season, the experimental room temperature ranged from 30 to 33°C. A 12 hours light/12 hours dark cycle with ad libitum food offered each day to an individual rats as well as fresh water (at normal temperature) were provided every day from 9-10 h (morning Rats were decapitated on day 7th (n=5) and day 28th (n=5) of experimental period from all groups to collected the blood in a hepranized tubes for the estimation of lymphocytes and neutrophils. Appropriate statistical analysis was performed to estimate the difference between age, days, treatments and their possible interactions during each season. During winter and summer seasons, male and female rats did show a significant decrease in lymphocytes, however a significant increase in the neutrophils percent was also observed in the HR and HDS groups. During summer, a significant increase in neutrophils and a decrease in lymphocytes were observed in male and female rats of HR and HDS groups.

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INTRODUCTION

The number of lymphocytes, eosinophils and monocytes decreased significantly in the flight animals; while neutrophils did increase at landing compared with preflight values (Allebban *et al.,* 1994). The total number of leukocytes, and absolute number of lymphocytes and monocytes decreased in flight rats and number of neutrophils did increase when compared with control rats (Ichiki *et al.*, 1996). During simulated weightlessness, the activity of lymphocyte decreased in human (Cogoli *et al.*, 1980). Total number of white blood cells rapidly increased in most crew members after spaceflight. This increase is due to an increase in the number of polymorph nuclear neutrophils, while the number of lymphocyte does not change or decrease significantly (Kimzey, 1977; Taylor *et al.*, 1986; Cogoli *et al.*, 1987). From all of the above reports, it can be concluded that variation in various cellular components of connective tissue is evident, however, alteration in leukocytes varies from one experiment to another one indicating that change is absolute but varies from one flight to another one in human as well as in experimental animals.

The chemical and physiological changes associated with microgravity can be investigated by using ground based models as a valuable tool, and the understanding about how microgravity plays its role in biochemical function and structure can be materially promoted (Woodman *et al*., 1991a, 1991b) There is an important role of ground based models to explore space as valuable data for the design of spaceflight experiment.

There is scarcity of data to compare the effects of season, age and sex in condition of simulated microgravity. Therefore, study was carried out to determine the effects of simulated microgravity induced by head down suspension on neutrophil and lymphocyte with age, gender and during winter and summer seasons.

MATERIALS AND METHODS

The effects of simulated microgravity were observed by using Albino rats (*Rattus norvegicus*) as animal model in the experiment. These rats were purchased from National Institute of Health, Islamabad, Pakistan. For the purpose of examination of any abnormalities or any pathological symptoms, the rats were kept under observation for two weeks. Only healthy rats in which no symptoms of sickness found were used during the study. The experiment was carried out in summer as well as during winter season. Rats matched with age and sex (n=240) were used in this study during winter and summer seasons. The minimum and maximum temperature during winter season was 20-22°C on day 7th, 21-23°C on day 14th, 21-23°C on day 21st and 20-23°C on day 28th while during summer it was 30-33°C on day 7th, 32-33°C on day 14th, 30-32°C on day 21st and 30-33°C on day 28th of experimental period. The average range temperature of the room was maintained according to the environmental condition in winter 20-23°C and for summer season 30-33°C respectively. Rats were divided into four experimental groups according to their sex and age (male and female rats, young and old). Every group consisted of 30 rats. Further each group was subdivided randomly into three groups i.e. cage control (CC), horizontal restraint (HR) and head down suspended (HDS). These subgroups had 10 rats in each. The experiment was carried out on light-dark cycle (12:12 hour) period. The composition of the feed offered to the control and experimental rats did have wheat (28.5%), fiber (28.5%), dry milk (23.0%), edible oil (5.0%), molasses (2.0%), fish meal (15.0%), salts (0.5%) and vitamin in small amount from ready made premix stock.

Suspension Procedure: For this purpose, the suspension model designed by Overton *et al.* (1989) was used. The model (35×35×30 cm) was made of Plexiglas, and at the top it was supported with a metal cross beam from which a harness apparatus was suspended. The rats were restrained within a semicircular Plexiglas tube by two Velcro straps, one located under the body posterior to the forelimbs and the other under the body anterior to the hind limbs. A hinged Plexiglas panel attached to semicircular tube was closed around the tail of the animal. HR rats were at 0° whereas HDS suspended at 45° in head down tilt position. To avoid the slip of animal from abdominal harness, its tail was secured in the panel by wrapping the cephalic half of the tail in a medicated adhesive tape. The distal half of the tail was monitored to authenticate that the process did not stop the blood flow to the tail. A bended metal bar supported the cross beam to which the harness was attached. This bended metal bar was designed to turn around the cross beam and allowed to adjust the height and angle of harness. The HDS rats were maintained individually in a 45° head down tilt position and hind limbs was non weight bearing. HDS rats moved freely using their forelimbs in 180° arc in their cages. A group of rats was horizontally restrained (HR) to examine the contribution of restraints to our findings. Rats of HR group were harnessed just like HDS, but not tilted and all limbs were weight bearing to move in a 180° arc within their cages. HR and HDS rats were maintained in similar cages and harness. The CC rats were kept as controlled and housed individually in metal cages. All rats in groups were offered food and water *ad libitum*.

Sampling Procedure: Half of the rats (n = 5) from each sub-group were used after 7 days; and the remaining half after 28 days of experimental period. Rats were weighed and then decapitated during 10:00 to 11:00 AM. Sterilized test tubes were used to collect blood using heparin as anticoagulant.

Hematology: A thin film was prepared from fresh blood, air dried, fixed with methanol and stained with Giemsa's stain (Benjamin, 1985). The stained area was covered by a cover glass slip by using Canada balsum. The oil immersion lens was used to make the examination of stained blood films for neurtrophils and lymphocytes.

Data collected was subjected to calculations of Mean + SE. Three way analysis of variance (Steel *et al.*, 1997) was adopted to analyze the data of male, female (gender), young, old (age), for winter and summer (seasons) for calculating the differences between control and treated rats for each parameters. For the statistical significance, $P<0.05$ and $P<0.01$ were considered statistically significant. In case of significant difference, Dunnett Multiple Range test was applied (Dunnett, 1955). Pearson's correlation was also estimated for each group within two seasons.

RESULTS

Neutrophils (%)

During winter: Overall mean neutrophils percentage was significantly high in old male rats as compared to young irrespective of their days of treatment (Table 1). Overall mean neutrophils percentage of male rats from HR and HDS did show a high percentage ($P\leq0.01$) by those of CC group during winter season (Fig. 1.I). On day 7, overall mean neutrophils were much higher in HR and HDS groups by those of CC group irrespective of their age (Fig. 1.II), but statistically non-significant.

Overall mean neutrophils percentage of young female rats did increase ($P\leq0.01$) from 7th to 28th days of treatment while in old female rats it did not differ significantly during this period (Table 1). Overall mean neutrophils percentage did increase ($P\leq0.01$) in HR and HDS treated female rats as compared to CC group (Fig. 1.III) while this increase was more prominent on 28th days of experimentation in these groups (Fig. 1.IV).

During summer: On day 7, mean neutrophils percentage in young male rats during summer season did increase ($P\leq0.01$) in HR and HDS groups as compared to CC and similar observations were recorded in old male rats (Table 1). On day 28th of experimental, HR and HDS groups of young and old male rats did show an increase ($P\leq0.01$) in neutrophil percentage but their neutrophils percentage was low ($P\leq0.01$) in young as compared to old rats (Table 1). Overall mean neutrophils did increase ($P\leq0.01$) in HR and HDS groups as compared to CC group (Fig. 1.V), however, neutrophils percentage was much higher in HR and HDS groups on 7th day of treatment (Fig. 1.VI) as compared to 28 days of treatment.

Table 1: Mean neutrophils (%±SE) of normal (CC), horizontal restrained (HR) and head down suspended (HDS) male and female rats at various days during winter and summer seasons

Season/ Days	Male						Female					
	Young			Old			Young			Old		
	CC	HR	HDS	CC	HR	HDS	CC	HR	HDS	CC	HR	HDS
Winter												
7	16.00 ±1.41	26.00 ±1.00	34.00 ±1.30	19.20 ±2.40	29.00 ±2.24	35.00 ±1.14	13.00 ±0.71	20.00 ±1.41	24.00 ±1.14	15.00 ±1.41	22.00 ±1.14	29.00 ±1.58
28	15.00 ±1.14	19.00 ±1.41	25.00 ±1.41	14.00 ±1.14	25.00 ±1.41	29.00 ±1.41	12.00 ±1.14	28.00 ±1.58	35.00 ±1.14	13.00 ±1.14	25.00 ±1.30	32.00 ±1.41
Summer												
7	16.00 ±0.70^{f}	27.00 ±1.14^{e}	38.00 ±1.00^{bc}	17.00 ±1.41^{e}	40.00 ±1.14^{b}	46.00 ±1.14^{a}	12.00 ±0.71	25.00 ±1.14	37.00 ±1.14	20.00 ±1.14	30.00 ±1.58	38.00 ±1.14
28	17.00 ±1.14^{f}	27.00 ±1.14^{e}	34.00 ±1.00^{cd}	18.00 ±0.71^{e}	32.00 ±1.40^{d}	37.00 ±1.00^{bc}	16.00 ±1.14	30.00 ±1.58	35.00 ±1.00	19.00 ±1.14	28.00 ±1.14	34.00 ±1.58

During summer a-e similar alphabets on means do not differ significantly at P≤0.01

Table 2: Mean lymphocytes (%±SE) of normal (CC), horizontal restrained (HR) and head down suspended (HDS) male and female rats at various days during winter and summer seasons

Season/ Days	Male						Female					
	Young			Old			Young			Old		
	CC	HR	HDS	CC	HR	HDS	CC	HR	HDS	CC	HR	HDS
Winter												
7	80.00 ±1.00	72.00 ±2.51	65.00 ±1.41	73.00 ±1.58	66.00 ±1.14	60.20 ±0.86	80.00 ±1.14	75.00 ±1.41	71.00 ±1.14	78.00 ±1.14	73.00 ±1.58	68.00 ±1.58
28	86.60 ±1.03	77.80 ±0.66	72.40 ±2.44	83.80 ±1.83	73.00 ±1.52	68.80 ±2.35	65.000 ±1.41	59.00 ±1.41	55.00 ±1.41	74.00 ±1.58	67.00 ±1.14	63.00 ±1.58
Summer												
7	78.00 ±1.14	68.80 ±1.36	58.60 ±2.62	75.60 ±1.63	56.60 ±1.21	56.60 ±1.21	80.40 ±1.03	71.20 ±1.16	59.00 ±0.71	70.60 ±1.72	64.20 ±1.56	58.00 ±1.14
28	76.40 ±1.72	70.00 ±1.82	64.00 ±0.71	80.00 ±0.71	67.20 ±0.86	60.00 ±1.58	80.00 ±0.71	65.40 ±1.33	53.00 ±1.14	71.80 ±1.16	67.60 ±2.02	61.00 ±1.41

Mean neutrophils of normal (CC), horizontal restrained (HR) and head down suspended (HDS) male rats during summer season are presented in table 1. Overall mean neutrophils percent was significantly high in young and old, HR and HDS rats as compared to CC group irrespective of their days of treatments (Table 1). Overall mean neutrophils percentage was high (P≤0.01) in female rats of HR and HDS groups as compared to CC group (Fig. 1.VII). On day 7th and 28th of experimental period, the percentage of neutrophils did increase (P≤0.01) in HR and HDS rats irrespective of their age (Fig. 1.VIII).

Lymphocytes (%)

During winter: Overall mean lymphocyte percent was higher (P≤0.01) in young male as compared to old male rats during winter (Table 1). Overall mean lymphocytes percent was significantly low in HR and HDS male rats as compared to CC group (Fig. 2.I). Overall mean lymphocytes did increase on 28th day of treatment in all groups but this was not significantly different from the values obtained at 7th day of the experimental period during winter irrespective of their age group (Fig. 2.II).

Old female rats did show an overall increase (P≤0.01) in lymphyocytes percent as compared to young female group irrespective of treatment and at different days of treatment (Table 1). Overall mean lymphocytes percent did decrease in HR and HDS female as compared to CC female rats irrespective of their age and days of treatment (Fig. 2.III). Overall mean lymphocytes percent in HR and HDS rats did decrease on day 7th and then on day 28th of experimental period but these differences was not significantly different (Fig. 2.IV).

During summer: Overall mean lymphocyte percentage was higher (P≤0.01) in young female as compared to old female rats irrespective of their days and treatment groups during summer (Table 1). Overall mean lymphocyte percent did decrease (P≤0.01) in HR and HDS groups as compared to CC group (Fig. 2. V). On day 7th and 28th of experimental period, the lymphocytes percent of female rats during summer did show decrease (P≤0.01) in HR and HDS group irrespective of their age (Fig. 2.VI)**.**

In young and old female rats, irrespective of their days of treatments, did show a significant decrease in overall mean lymphocytes percent in HR and HDS groups during summer (Table 1). Overall mean lymphocyte percent of female rats during summer did decrease (P≤0.01) in HR and HDS group when compared with CC group (Fig. 2.VII). This decrease however was not significant on 7th and 28th day of experimental period irrespective of their age (Fig. 2.VIII).

DISCUSSION

Neutrophils: During winter, no significant change in neutrophil numbers was observed in all experimental groups of young as well as old rats. However, in summer, neutrophils did increase in HR and HDS groups in young and old rats. Rats in HR and HDS groups during winter did increase their neutrophils and were significantly higher in HDS group as compared to HR rats. Almost, similar observations were observed in HR and HDS rats during summer season. On day 7th, during winter, neutrophils number did increase in male, while in female, neutrophils number increase in significantly in HR and HDS groups and this trends was persistent even on the 28th day of experimental period.

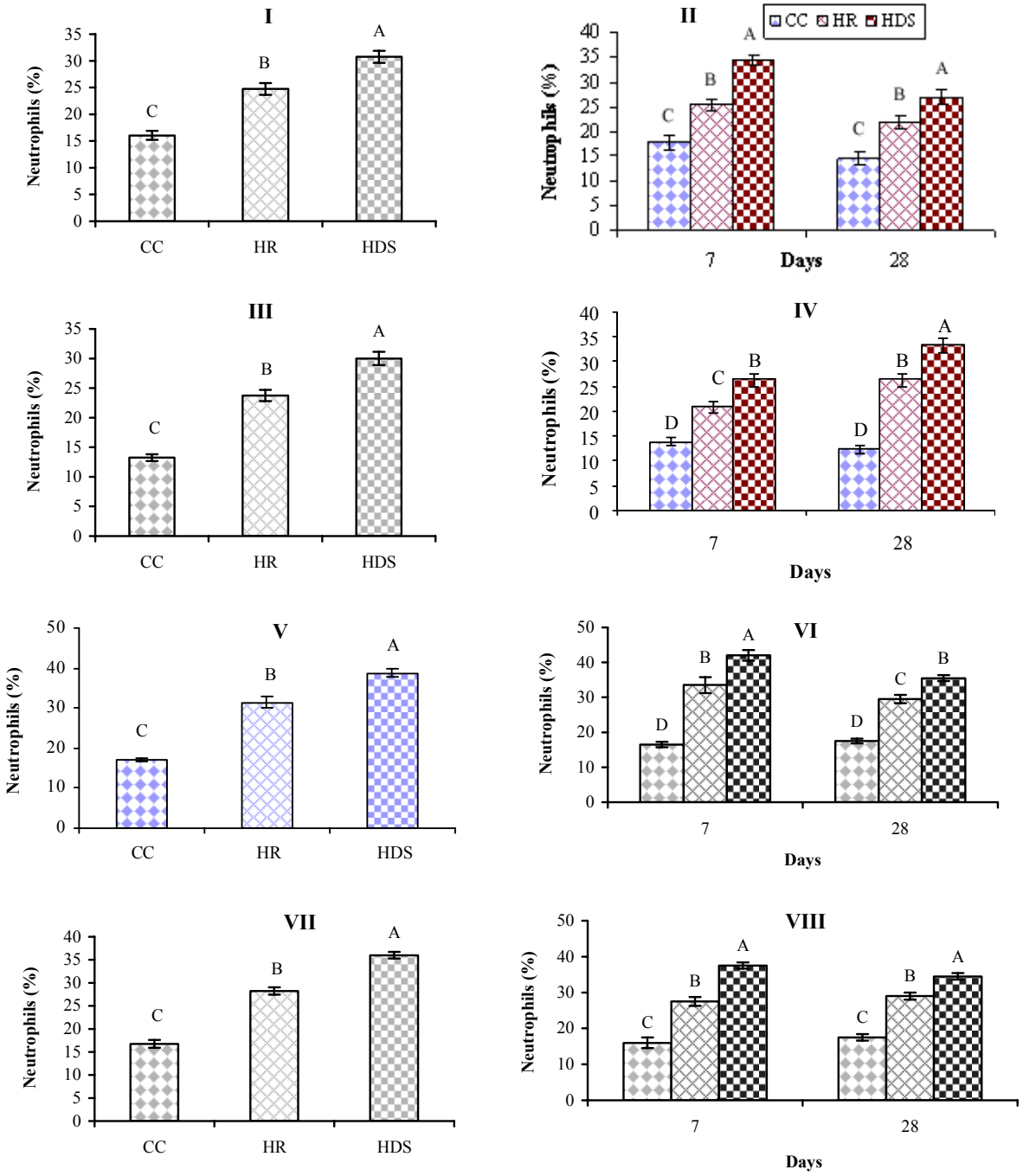

Fig. 1: Overall mean neutrophils (%±SE) of normal (CC), horizontal restrained (HR) and head down suspended (HDS) male rats during winter (I), at different days (II), and in female rats during winter (III), at different days (IV) of treatments. Overall mean neutrophils (%±SE) from CC, HR and HDS groups has also been shown for male (V), at different days (VI), and in female (VII), at different days (VIII) during summer season.

During summer, in male and female rats, neutrophils were significantly high in HR and HDS groups on day 7th and 28th of experiment. Overall, a significant increase in neutrophils was observed in HDS group as compared to HR during winter and summer and in young and old rats during the present study. Allebban *et al.* (1994) reported a slight increase in the absolute number of neutrophils while a slight decrease was also observed in the absolute number of eosinophils. On the other hand, Bechler *et al.* (1986) and Cogoli *et al.* (1987) did show a quick rise in total blood cell count due to an increase number of polymorphonucler neutrophils, while lymphocytes count did not change. In the present study, increase in neutrophils in HR and HDS groups during winter and summer did show that stress on one hand depressed the lymphocytes number and as well as might alter their functions.

No significant correlation was observed during winter seasons, however, neutrophil was correlated (r = 0.463) with body weight during summer. Neutrophil of HR groups did show negative correlation with pituitary (r = –0.345) during winter and with adrenal (r = –0.388), liver

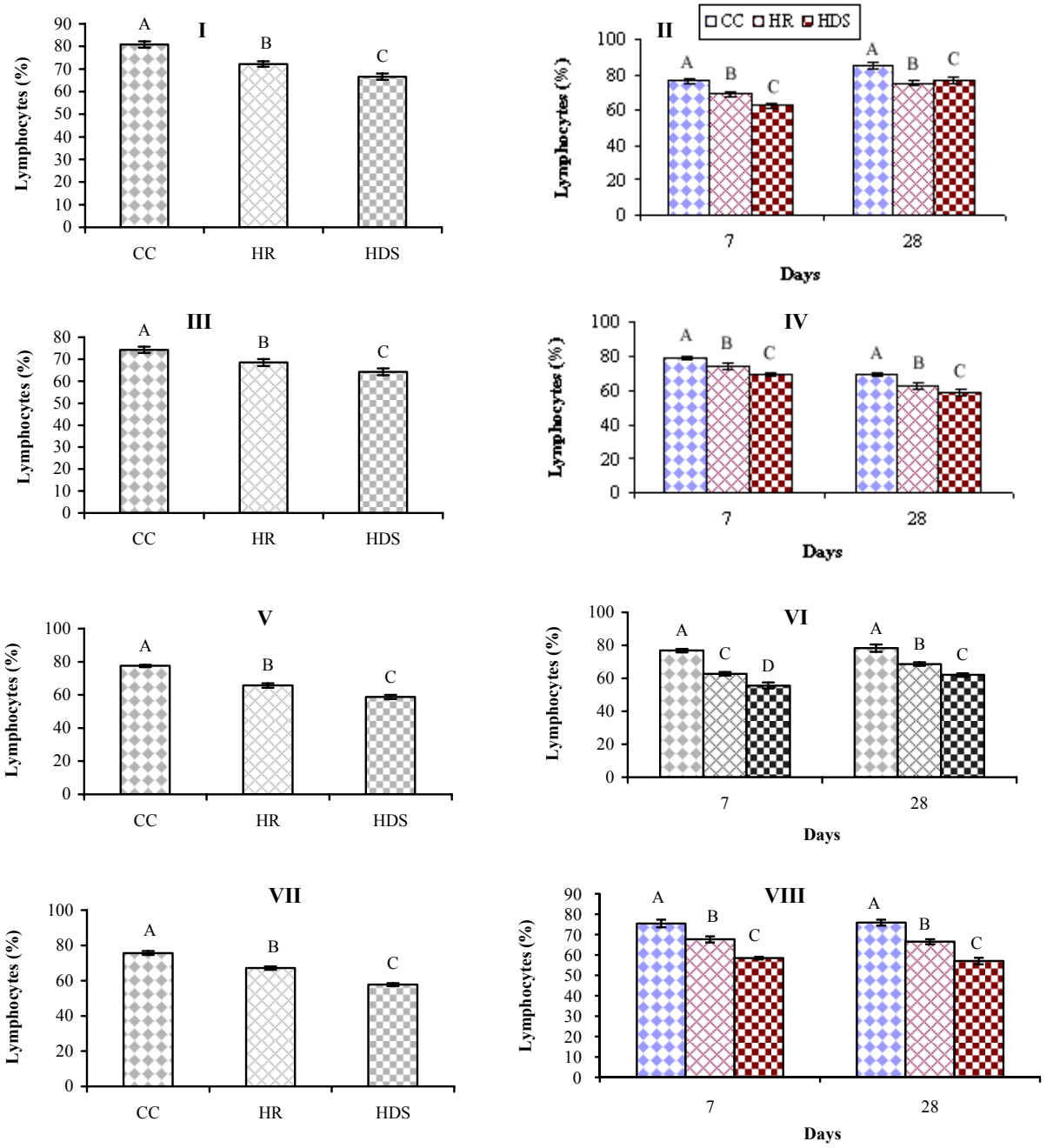

Fig. 2: Overall mean lymphocytes (%±SE) of normal (CC), horizontal restrained (HR) and head down suspended (HDS) male rats during winter (I), at different days, (II) and in female rats during winter (III), at different days(IV) of treatments. Overall mean lymphocytes (%±SE) from CC, HR and HDS groups has also been shown for male (V), at different days (VI) and in female (VIII), at different days (VIII) during summer season.

(r = –0.443) and pituitary (r = –0.418) a negative correlation during summer. Rats neutrophils from HDS groups did show a positive correlation with liver (r = 0.488) during winter.

Lymphocytes: Lymphocytes during winter season did not change in all experimental rats observed in CC, HR and HDS groups. During summer, lymphocyte were significantly low in HR as compared to CC group and significantly high in HDS group as compared to HR group in male and female of young and old rats. In HR and HDS groups, both male and female rats did show a decrease ($P \geq 0.01$) in lymphocytes during winter and summer seasons. Modifications in immune cells has been reported by Tipton *et al.* (1996), Taylor *et al.* (1997), Levine and Greenleaf (1998), Borchers *et al.* (2002) and Crucian *et al.* (2008) indicated that total white blood cells, lymphocytes and monocytes were decreased significantly as compared to control rats during space flight. Baqai *et al.* (2009) observed a significant space induced decrease in lymphocytes, monocytes and macrophages. A vital impact of space flight environment on a variety of immune response was also reported by several authors (Nash and Mastro, 1992; Crucian *et al.*, 2000).

Lymphocyte from control rats did show a positive correlation with body weight (r = 0.493) during winter. Like wise during summer lympocytes showed a negative correlation with body weight (r = –0.529) and neutrophils (r = –0.549) indicating that normal rats were influenced with seasons. In rats of HR group, a positive correlation was observed with body weight (r = 0.432) and kidney (r = 0.316) and negative relationship with neutrophil (r = –0.502) during winter while during summer, lymphocyte were correlated with liver (r = 0.391) positively and with neutrophils (r = –0.633) negatively indicating that HR groups not only behaved differently within the group but also during two different seasons. Lymphocytes in HDS rats were negative correlated with liver (r = –0.470) and neutrophils (r = –0.704) during winter and with neutrophils (r = –0.485) during summer. It is very much evident that during summer lymphocytes and neutrophils did decrease in normal, HR and HDS groups while during winter this decrease was observed only in HR and HDS groups.

In conclusion, during winter and summer neutrophils did increase in male and female rats but these differences were only significant in male as compared to female rats. On the other hand, lymphocytes did decrease significantly in male and female rats of HR and HDS groups during winter and summer. At different days of treatment male rats of HR and HDS groups during winter did show an increase in neutrophils. Female rats during winter and male and female rats of HR and HDS groups during summer did show a significant increase in neutrophils as compared to control. Lymphocytes percentage did decrease in male and female rats of HR and HDS groups during winter and in female rats during summer as compared to control on 7^{th} and 28^{th} days of treatment. Male rats of HR and HDS groups during summer did show a significant decrease in lymphocytes as compared to their control rats. Between groups significant increase or decrease of neutrophils and lymphocytes was more profound in HDS group as compared to HR group.

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