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Duration dependent effect of chronic stress on primary and secondary lymphoid organs and their reversibility in rats

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ABSTRACT

The present study was undertaken to investigate whether or not chronic stress effect and its reversibility on lymphoid organs is duration dependent. Male rats were exposed to restraint (1 h) followed by a gap of 4 h to forced swimming exercise (15 min) daily for 2, 4 and 8 weeks. After each exposure period, rats were allowed to recover for 6 weeks. Stress exposure resulted in duration dependent decreases in weight of thymus and axillary lymph nodes, lymphocyte counts of spleen, thymus and axillary lymph nodes and number of islets of white pulp of spleen and increases in apoptotic index of splenocytes, thymocytes and lymphocytes of axillary lymph nodes. All the parameters of lymphoid organs studied showed significant alterations in 2 weeks of stress exposure indicated their sensitivity to stress effects in short term exposure and thymus was the most sensitive organ among all. The alterations in all the parameters of spleen and majority of parameters of thymus and axillary lymph nodes returned to control level in recovery group rats of 2 and 4 weeks exposure but not in that of 8 weeks exposure. The present study for the first time reveal that severity of stress effects on lymphoid organs increases with increasing duration of exposure and shorter the exposure period faster the recovery. In addition, an *in vitro* study showed that corticosterone caused apoptosis of thymocytes, splenocytes and lymphocytes of axillary lymph nodes in dose dependent manner. Thus corticosterone induced death of cells of lymphoid organs under stress is the major cause of involution of lymphoid organs.

1. Introduction

Stressful conditions activate the hypothalamic–pituitary–adrenocortical axis (HPA) and sympathetic nervous system that results in excessive secretion of stress related neuro-endocrine mediators (Padgett and Glaser, 2003). The neuro-endocrine mediators affect the cells of the primary and secondary lymphoid organs and bring alterations in immune functions (Sabioncello et al., 2004). The lymphoid organs are the specialized tissues of the immune system, where in lymphocytes are generated in primary lymphoid organs (bone marrow and thymus) and then transferred to secondary lymphoid organs (spleen, lymph nodes *etc.*) (Ruddle and Akirav, 2009). Proper maturation and distribution of these lymphoid cells are essential for the generation of strong immune responses. Secondary lymphoid organs are the sites where mature lymphocytes are stimulated to respond to invading pathogens. Functionally mature lymphocytes continuously circulate between blood and secondary lymphoid organs in search of pathogens and evoke adaptive immunity which is helpful in the termination of infection (Parham, 2014).

Different types of stressors alter this distribution pattern of lymphocytes between lymphoid organs and blood (Hoffman-Goetz et al., 1989; Dominguez-Gerpe and Rey-Mendez, 2001). A few *in vivo* studies have reported the effect of stress on lymphoid organs and showed reduction in weight, cellularity and alteration in histological architecture (Morishita et al., 1997; Dominguez-Gerpe and Rey-Mendez, 2001; Stefanski et al., 2003; Hernandez et al., 2013; Srinivasan et al., 2016; Sarjan et al., 2017). However, these studies did not focus whether stress induced reduction in cellularity are due to altered distribution of lymphoid cells or due to cell death. In addition, a few *in vitro* studies (Nieto et al., 1992; Vicent et al., 2000; Yun-Feng and Zhi-Pu, 2002) showed corticosterone induced apoptosis of thymocytes, splenocytes and pheochromocytoma cells. However, there are no studies where in both *in vivo* and *in vitro* experiments were combined to analyze the effect of endogenous as well as exogenous corticosterone on cells of lymphoid organs. More ever, each *in vitro* study used pharmacological doses of corticosterone to demonstrate cell death (Vicent et al., 2000; Yun-Feng and Zhi-Pu, 2002). Hence, there is a need to find out the effects of stress level concentration of corticosterone on immune cells in *in vitro* to understand the stress effects on lymphoid organs. Further, the studies conducted so far focused on stress effects on any

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one of primary or secondary lymphoid organs (Pronin et al., 1997; Tournier et al., 2001; Zivkovic et al., 2005; Hernandez et al., 2013) and therefore differential sensitivity of primary and secondary lymphoid organs to stress exposure and stress mediators could not be ascertained by these studies.

In addition, the effect of stress on primary and secondary lymphoid organs was studied either under short term (Tarcic et al., 1998; Tournier et al., 2001; Archana, 2013; Moazzam et al., 2013; Srinivasan et al., 2016) or long term (Hernandez et al., 2013; Divyashree et al., 2016) exposure. Since these studies were conducted for a single duration of stress exposure it is not possible to determine whether the damage in lymphoid organs increases with increasing duration of exposure from these studies. In addition, a single study was conducted on reversibility of stress effects on lymphoid organs following a single duration of stress exposure (Divyashree et al., 2016). However, the study did not focus whether reversibility of stress induced damage on lymphoid organs depends on duration of stress exposure. In the view of these lacunae, the proposed study was conducted to find out whether sensitivity of chronic stress induced effects on different lymphoid organs differ with increase in duration of exposure and if so whether reversibility depends on duration of exposure. Further, dose dependent effects, if any of corticosterone on apoptosis of cells of different lymphoid organs has also been investigated.

2. Materials and methods

2.1. Animals

Adult male albino rats weighing 200–220 g were obtained from the inbred colony of the central animal facility of the University of Mysore. The rats were maintained under 12:12 h light and dark cycle and were supplied with standard rat chow and water *ad libitum*. All procedures performed in the studies involving animal participants were in accordance with the ethical standards of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India. Approval for the proposed animal experiments was obtained from the Institutional Animal Ethical Committee of University of Mysore, India (Reference number UOM/IAEC/ 07/2016).

2.2. Procedure for inducing stress

Two types of stressors were used to induce stress (Grissom et al., 2008).

(1) Restraint: a rat was placed in cylindrical restrainer of 6.7 cm diameter and 22.2 cm length for 1 h.
(2) Forced swimming exercise: rats were forced to swim in a glass chromatography jar (18 in. height × 8.75 in. outer diameter) filled two third full of water for 15 min at room temperature.

2.3. Experimental design

2.3.1. In vivo experiment

Adult male rats were randomly divided into control (n = 15) and stress (n = 30) groups. The control rats were maintained without any disturbance whereas rats in stress group were subjected to restraint (1 h) followed by forced swimming exercise (15 min) after a gap of 4 h every day for 2, 4 and 8 weeks. After each treatment period, five rats from control as well as stress group were sacrificed and five rats in stress group were maintained without exposing to stressors as recovery group for 6 weeks and then sacrificed.

At autopsy, spleen, thymus and axillary lymph nodes were removed, cleared of fat, weighed and cells were isolated for apoptosis assay and isolation of DNA. The absolute organ weight of each lymphoid organ was converted into relative organ weight (weight/100 g body weight). The excised lymphoid organs were fixed in Bouin's fluid for histological studies.

2.3.1.1. Isolation of splenocytes, thymocytes and lymphocytes of axillary lymph nodes. The spleen, thymus and axillary lymph nodes were pressed through 400 µm sterile nylon mesh to harvest splenocytes, thymocytes and lymphocytes of axillary lymph nodes respectively. Cell suspension was centrifuged at 2000 rpm at 4 °C for 5 min and cell pellet was collected. Cell pellet was washed and suspended in the HBSS medium and cellularity was determined by using Neubauer counting chamber with the help of Giemsa (Zager et al., 2007).

2.3.1.2. Apoptosis assay. Ten microlitre each of ethidium bromide, acridine orange and one of the cell suspensions of splenocytes, thymocytes and lymphocytes of axillary lymph nodes were mixed and loaded in Neubauer counting chamber and allowed to stand. Cells were observed under fluorescent microscope (Zeisss Imager, A2, camera AxioCamMRc 5) using 450 nm and 530 nm filters, where, healthy cells appeared green and apoptotic cells as red (Tatlican et al., 2009). Randomly selected 500 cells per rat sample of each organ were observed and number of healthy (green) and apoptotic (red) cells was recorded (Grossmann et al., 1998):

Apoptotic index = (Number of apoptotic cells/total number of cells counted) × 100

2.3.1.3. DNA ladder assay. Genomic DNA was extracted from the cells of spleen, thymus and axillary lymph nodes by phenol/chloroform method and DNA was separated by agarose gel (0.8%) electrophoresis. The gel was photographed under ultraviolet illumination (Biase et al., 2002).

2.3.1.4. Estimation of serum concentration of corticosterone. Serum concentration of corticosterone was quantified using ELISA kit manufactured by Demeditec diagnostics GmbH, Germany and the procedure of the manufacturer was adopted.

2.3.1.5. Histological study. Five micron thick paraffin sections of spleen, thymus and axillary lymph nodes were cut and stained with hematoxylin and eosin for histological study and significant changes were photographed. In the spleen, number of islands of white pulp was counted from 50 randomly selected cross sections per rat of each experimental group.

2.3.2. In vitro experiment

An *in vitro* study was conducted to assess the effect of corticosterone on splenocytes, thymocytes and lymphocytes of axillary lymph nodes. Cells of spleen, thymus and axillary lymph nodes were isolated and a cell suspension was prepared as described above. Hundred microlire of cell suspension (2×10^7) was incubated at 37 °C in 1 ml of RPMI 1640 medium containing one of the different concentrations of corticosterone (100, 200, 300, 400 and 500 ng/ml of medium) and 10 µl each of fetal calf serum, penicillin and streptomycin. After the incubation, the cells were gently re-suspended and centrifuged at 2000 rpm and cell pellets were used for apoptosis assay as described in the above Section 2.3.1.2.

2.4. Statistical analysis

The mean ± SE of each parameter was computed considering the data on 5 rats per group and mean values of each parameter of different groups were compared using one way ANOVA followed by Duncan's multiple range test and judged significant if $P < 0.05$.

Table 1
Effects of chronic stress on body weight, weight of spleen, thymus and axillary lymph nodes and serum concentrations of corticosterone in rats.

Groups	Body weight Mean (g) ± SE	Serum corticosterone concentration (ng/ml) Mean ± SE	Mean weight (mg)/100 g body weight ± SE		
			Spleen	Thymus	Axillary lymph nodes
2 weeks					
Control	201.40 ± 6.10^{b}	105.22 ± 3.31^{a}	277.80 ± 11.26^{a}	76.34 ± 8.73^{a}	63.78 ± 4.05^{a}
Stress	181.25 ± 4.26^{c} (−10.00%)	340.49 ± 6.65^{b} (+223.60%)	222.38 ± 5.72^{b} (−19.95%)	48.89 ± 6.88^{cd} (−35.96%)	40.29 ± 6.57^{c} (−38.83%)
Recovery	201.00 ± 1.41^{b}	123.28 ± 4.84^{a}	268.33 ± 7.69^{a}	69.88 ± 1.33^{ab}	57.64 ± 4.93^{ab}
4 weeks					
Control	204.75 ± 1.84^{b}	110.07 ± 5.70^{a}	292.53 ± 17.31^{a}	75.92 ± 5.33^{a}	62.65 ± 3.83^{a}
Stress	182.75 ± 6.65^{c} (−10.74%)	400.83 ± 7.03^{c} (+264.16%)	219.94 ± 3.25^{b} (−24.81%)	45.79 ± 1.01^{cd} (−39.69%)	34.56 ± 1.74^{c} (−44.85%)
Recovery	207.00 ± 10.69^{b}	123.49 ± 5.76^{a}	272.39 ± 29.33^{a}	45.91 ± 5.71^{cd}	58.58 ± 5.42^{ab}
8 weeks					
Control	226.80 ± 4.00^{a}	107.16 ± 3.10^{a}	286.78 ± 25.42^{a}	56.39 ± 5.52^{bc}	69.10 ± 8.58^{a}
Stress	185.00 ± 3.68^{c} (−18.43%)	484.23 ± 9.07^{d} (+351.88%)	219.05 ± 3.11^{b} (−23.62%)	32.28 ± 6.38^{d} (−42.76%)	33.00 ± 3.07^{c} (−52.17%)
Recovery	184.33 ± 6.28^{c}	388.72 ± 5.42^{c}	214.45 ± 7.64^{b}	31.38 ± 5.03^{d}	43.19 ± 5.70^{bc}
ANOVA F Value (df = 8, 36)	9.01 P < 0.001	725.01 P < 0.001	5.20 P < 0.001	9.95 P < 0.001	5.85 P < 0.001

Note: Number in parenthesis in each column indicates % change compared to controls, + denotes increase, − denotes decrease. Groups with same superscript letters do not significantly differ among themselves, whereas groups with different superscript letters significantly differ. df: degree of freedom.

Table 2
Effects of chronic stress on lymphocyte counts in different lymphoid organs of rats.

Groups	Number of cells X 10^{6}/ml (mean ± SE)		
	Splenocytes	Thymocytes	Lymphocytes of axillary lymph nodes
2 weeks			
Control	734.33 ± 15.30^{a}	271.33 ± 8.17^{a}	104.37 ± 45.66^{a}
Stress	612.00 ± 6.65^{bc} (−16.66%)	194.66 ± 2.40^{b} (−28.26%)	80.00 ± 18.92^{bc} (−23.35%)
Recovery	729.33 ± 26.10^{a}	257.20 ± 8.62^{a}	100.66 ± 80.63^{a}
4 weeks			
Control	726.66 ± 4.63^{a}	280.62 ± 20.96^{a}	104.30 ± 6.18^{a}
Stress	562.33 ± 34.37^{c} (−26.61%)	188.62 ± 15.52^{b} (−32.78%)	73.02 ± 5.06^{c} (−29.99%)
Recovery	666.20 ± 26.82^{ab}	249.33 ± 11.03^{a}	93.60 ± 3.84^{b}
8 weeks			
Control	726.50 ± 36.51^{a}	284.00 ± 5.56 ^{a}	101.33 ± 4.41^{a}
Stress	530.33 ± 15.98^{c} (−27.53%)	179.60 ± 15.17^{b} (−36.76%)	68.90 ± 7.00^{c} (−32.00%)
Recovery	580.00 ± 23.57^{c}	185.40 ± 8.49^{b}	77.00 ± 9.12^{bc}
ANOVA F Value (df = 8, 36)	9.07 P < 0.001	8.36 P < 0.001	6.21 P < 0.001

Note: Number in parenthesis in each column indicates % change compared to controls, + denotes increase, − denotes decrease. Groups with same superscript letters do not significantly differ among themselves, whereas groups with different superscript letters significantly differ. df: degree of freedom.

3. Results

3.1. In vivo experiment

3.1.1. The body weight and serum corticosterone concentration

The body weight of rats exposed to stress for 2, 4 and 8 weeks showed a significant decrease compared with that of respective controls. In the recovery group rats after 2 or 4 weeks stress, body weight was significantly higher than that of stress groups, whereas in recovery group after 8 weeks exposure it was significantly lower than controls and was similar to stress group. The Serum concentration of corticosterone was significantly increased in stress group rats of all the three durations compared to controls. However, in recovery groups after 2 and 4 weeks stress, serum corticosterone concentration was similar to that of controls but not in recovery group rats after 8 weeks stress. The percentage increase in serum levels of corticosterone and the percentage decrease in body weight showed significant changes in a pattern, control < 2 weeks stress < 4 weeks < 8 weeks and 8 weeks stress > 4 weeks > 2 weeks > controls respectively (Table 1).

3.1.2. The relative weight of lymphoid organs

The relative weight of the spleen, thymus and axillary lymph nodes of rats exposed to stress for 2, 4 and 8 weeks showed a significant decrease compared with that of respective control groups. The relative weight of the spleen and axillary lymph nodes in recovery groups following 2 and 4 weeks stress did not differ from respective controls whereas that of recovery rats after 8 weeks did not differ from stress group. The relative weight of thymus of recovery group after 2 week stress did not differ from controls, whereas after 4 and 8 weeks stress did not significantly differ respective stress groups. The percentage decrease of relative weight of thymus and axillary lymph nodes followed a pattern, control > 2 weeks > 4 weeks > 8 weeks stress (Table 1).

3.1.3. Counts of cells of spleen, thymus and axillary lymph nodes

Counts of splenocytes, thymocytes and lymphocytes of axillary lymph nodes were significantly lower in stressed rats of all durations of exposure compared to respective controls. In the recovery group rats, cellularity of spleen and thymus was restored to control levels following 2 and 4 weeks stress exposure but not after 8 weeks stress exposure. However, lymphocyte count of axillary lymph nodes was restored to control levels in recovery rats after 2 weeks stress but not following 4 and 8 weeks exposure. The percentage decrease in cells of spleen, thymus and axillary lymph nodes showed a pattern, 8 weeks > 4 weeks > 2 weeks stress > controls (Table 2).

3.1.4. Apoptotic assay

More number of apoptotic cells (red) and less number of healthy cells (green) were observed in stressed rats of all durations compared to controls in all cell types of lymphoid organs (Fig. 1). A significant increase in apoptotic index of cells of lymphoid organs was observed in stressed rats of all the three durations of exposure compared to controls. The apoptotic index of splenocytes, thymocytes and lymphocytes of axillary lymph nodes of recovery group rats following 2 and 4 weeks stress didn't differ from controls whereas that of recovery rats after 8 weeks stress was significantly lower than stressed rats and it was

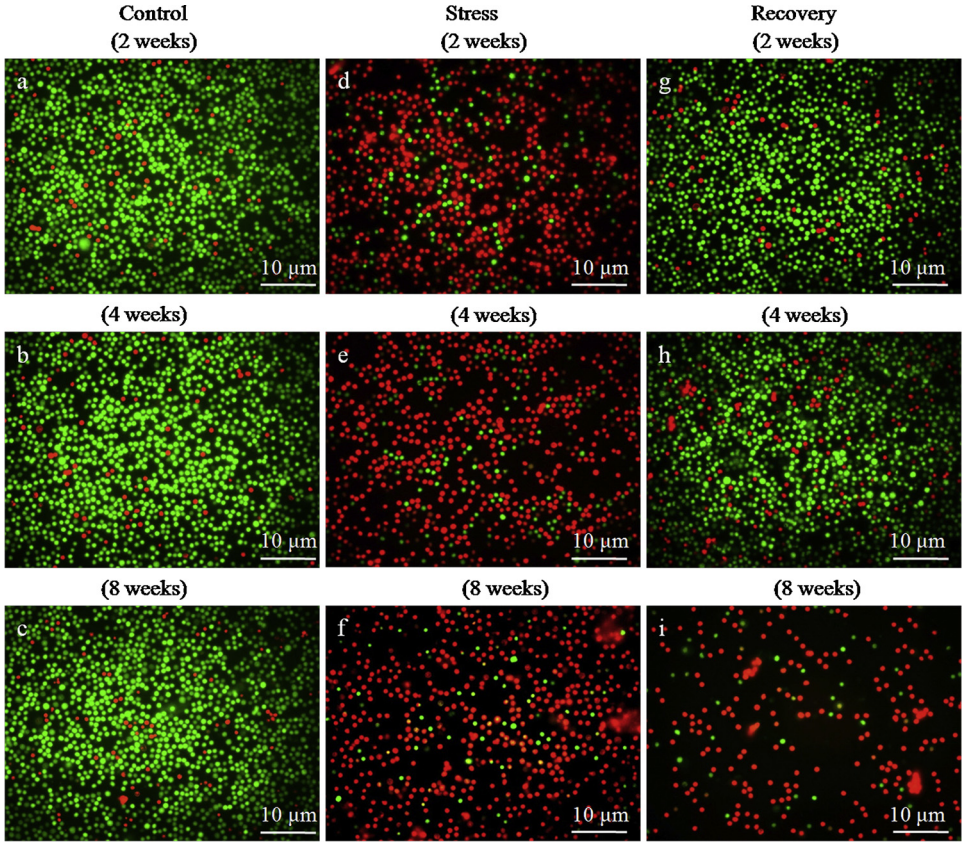

Fig. 1. Photomicrographs showing florescent staining of splenocytes (a–i). The florescent staining involved staining of cell suspensions with acridine orange and ethidium bromide (450 nm and 530 nm). Note the presence of more number of healthy cells (green) in controls (a–c), more number of apoptotic cells (red) in stressed rats (d–f) and more number of healthy cells (green) in recovery group rats after 2 weeks (g) and 4 weeks (h) stress whereas more number of apoptotic cells (red) in recovery group rat (i) after 8 weeks stress. 400×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Effects of chronic stress on apoptotic index of cells of lymphoid organs of rats.

Groups	Mean apoptotic index of cells ± SE		
	Splenocytes	Thymocytes	Lymphocytes of Axillary lymph nodes
2 weeks			
Control	13.33 ± 1.20^{a}	14.60 ± 1.32^{a}	14.00 ± 0.70^{a}
Stress	26.33 ± 0.33^{bc} (+97.33%)	36.60 ± 3.55^{cd} (+150.68%)	38.66 ± 2.33^{bc} (+176.14%)
Recovery	16.75 ± 2.21^{ab}	23.80 ± 4.44^{ab}	20.00 ± 3.89^{a}
4 weeks			
Control	14.33 ± 2.90^{a}	15.40 ± 3.74^{a}	16.20 ± 2.08^{a}
Stress	32.66 ± 2.02^{cd} (−127.91%)	40.33 ± 2.40^{cd} (+161.88%)	47.33 ± 5.04^{cd} (+192.16%)
Recovery	17.25 ± 3.30^{ab}	23.00 ± 4.24^{ab}	22.00 ± 3.31^{a}
8 weeks			
Control	14.00 ± 2.64^{a}	13.00 ± 2.98^{a}	15.40 ± 2.11^{a}
Stress	38.25 ± 5.05^{d} (-173.21%)	45.33 ± 2.33^{d} (+248.69%)	50.33 ± 5.20^{d} (+226.82%)
Recovery	32.00 ± 5.19^{cd}	32.00 ± 2.64^{bc}	32.66 ± 5.66^{b}
ANOVA F Value (df = 8, 36)	8.70 P < 0.001	10.49 P < 0.001	17.16 P < 0.001

Note: Number in parenthesis in each column indicates % change compared to controls, + denotes increase, − denotes decrease. Groups with same superscript letters do not significantly differ among themselves, whereas groups with different superscript letters significantly differ. df: degree of freedom.

significantly higher than controls. The percentage increase in apoptosis of cells of lymphoid organs showed the pattern, control < 2 weeks < 4 weeks < 8 weeks stress (Table 3).

3.1.5. DNA ladder assay

Fragmentation of DNA in splenocytes, thymocytes and lymphocytes of axillary lymph nodes was found only in 8 weeks exposure to stressors

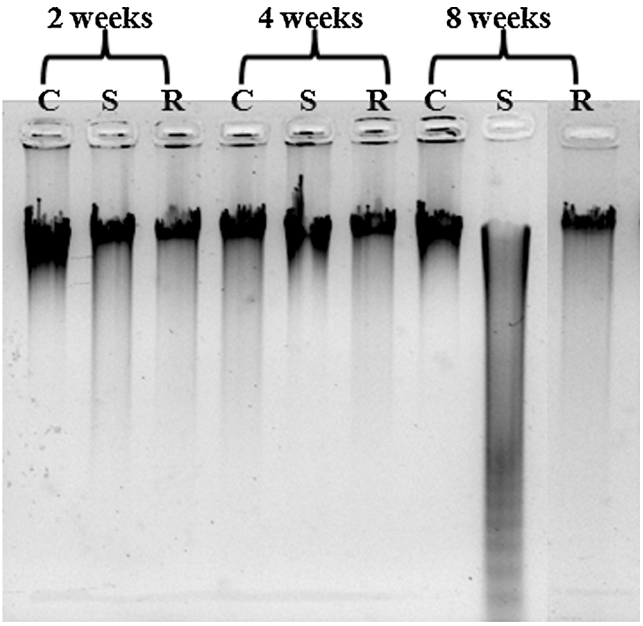

Fig. 2. Photomicrographs of DNA gel electrophoretic profile of splenocytes of control, rats exposed to stress for 2, 4 and 8 weeks and recovery group rats. Note the presence of DNA breaks in splenocytes of rats exposed to stress for 8 weeks but not in control, recovery rats. Note the absence of DNA breaks in rats exposed to 2 and 4 weeks stress. C, control lane; S, stress lane; R, recovery lane.

compared to controls as shown by the ladder like appearance of DNA following electrophoresis (Fig. 2). In recovery groups after 8 weeks stress, no breaks were found in DNA of splenocytes, thymocytes and lymphocytes of axillary lymph nodes.

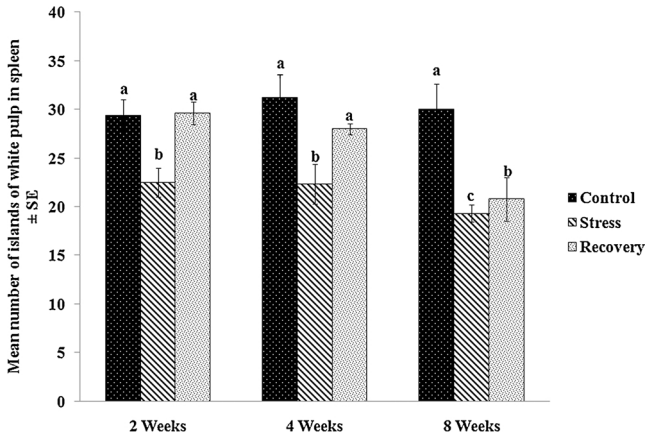

Fig. 3. Vertical bars showing mean number of islands of white pulp of spleen in control, stress and recovery group of rats. Bars with same superscript letters do not significantly differ among themselves whereas those with different superscript letters significantly differ.

3.1.6. Histomorphology of spleen, thymus and axillary lymph nodes

The spleen consists of two distinct compartments, the red pulp and the white pulp. Red pulp contained red blood cells, macrophages and plasma cells. The white pulp showed actively dividing lymphocytes and central arteries. The number of islands of white pulp in spleen was significantly decreased in stressed rats in all three durations compared to respective controls. In recovery groups after 2 and 4 weeks stress exposure, the number of islands did not significantly differ from controls, whereas in recovery group rats after 8 weeks stress it was similar to that of stress group (Fig. 3). A reduction in germinal centre and shrinkage of white pulp were found in stressed rats compared to respective control rats. In recovery group rats after 2 and 4 weeks exposure, histological architecture was similar to that of control rats. However, in recovery group rats after 8 weeks exposure histological alterations were similar to that of stress group (Fig. 4).

Thymic cortex consists of rapidly dividing densely packed T lymphocytes and a few macrophages. The lighter-staining medullary areas contained fewer lymphocytes and Hassall's corpuscles. There was no differentiation between cortex and medulla of thymus of 2, 4 and 8 weeks stressed rats and showed the development of connective tissue and fat cells. In the recovery group after 2 weeks exposure, histology of thymus was similar to that of controls, whereas, recovery group rats after 4 and 8 weeks exposure resembled stressed rats (Fig. 5).

Lymph node had two distinct regions, the cortex and the medulla. The superficial part of the cortex contained densely packed follicles with germinal centers and dividing B lymphocytes. Medullary cords were thin inward extensions from the cortical lymphoid tissue and contained lymphocytes, plasma cells, lymph sinuses and large lymph capillaries. Shrinkage of the germinal centre of lymphoid follicles was observed in stressed rats of all durations of exposure compared to control rats. In the recovery group rats after 2 weeks exposure, the lymph nodes resembled controls, whereas, those after 4 and 8 weeks exposure it was similar to stressed rats (Fig. 6).

3.2. In vitro experiment

3.2.1. Apoptosis assay

3.2.1.1. Splenocytes. A significant dose dependent increase in the apoptosis of splenocytes was observed following treatment with 100, 200, 300 and 500 ng/ml concentrations of corticosterone compared to

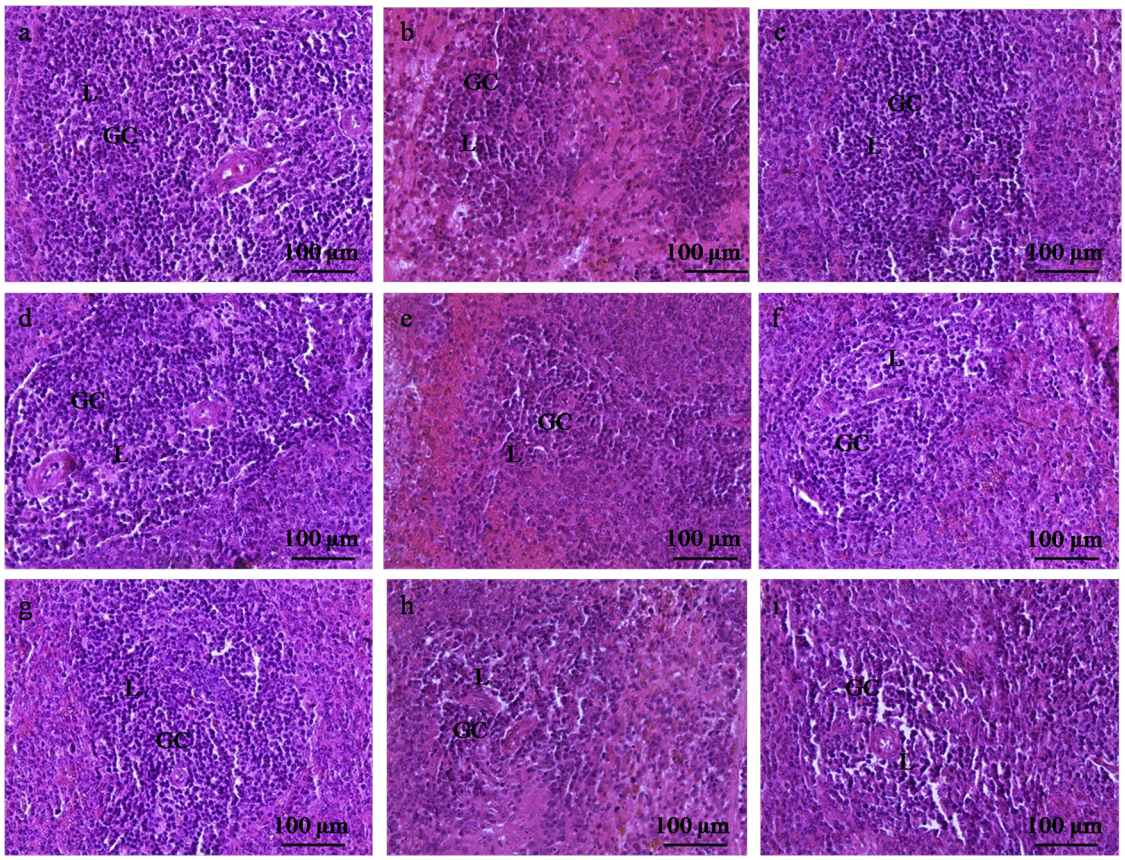

Fig. 4. (a–i) Photomicrographs of the cross sections of the spleen showing the island of white pulp. Note the shrinkage of island of the white pulp region and germinal centre in stressed rats of 2 weeks (b), 4 weeks (e) and 8 weeks (h), recovery rats of 8 week (i) compared to controls of 2 weeks (a), 4 weeks (d) and 8 weeks (g) and recovery rats of 2 weeks (c) and 4 weeks (f). 200× (H&E). GC: germinal center; L: lymphocytes.

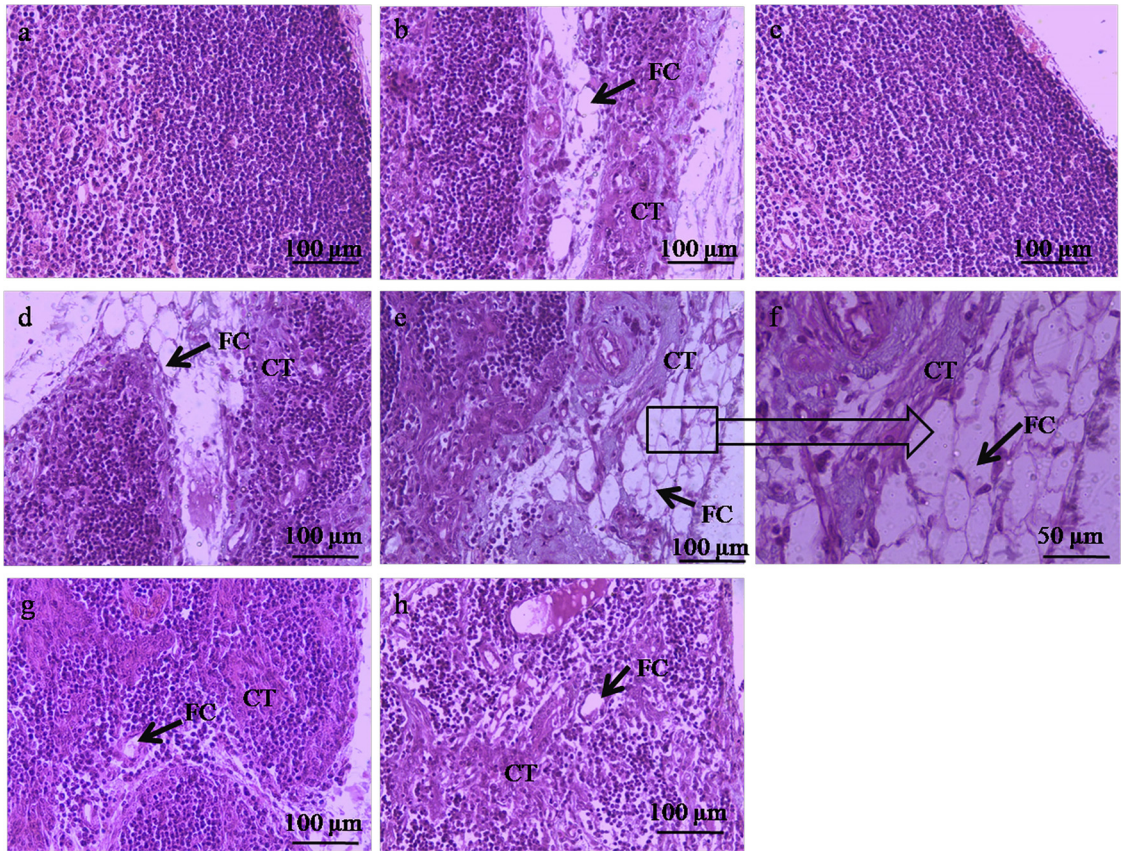

Fig. 5. (a–h) Photomicrographs of the cross sections of thymus gland. Note the disorganization of cortical and medullary region, development of connective tissue and fat cells in stressed rats of 2 weeks (b), 4 weeks (d) and 8 weeks (e and f), recovery group rats after 4 weeks (g) and 8 weeks (h) stress compared to controls of 2, 4 and 8 weeks (a) duration and recovery group after 2 weeks stress (c). 200X (H&E). CT: connective tissue; FC: fat cells.

controls. However, apoptotic index at 400 ng concentration did not significantly differ either from 300 ng or 500 ng concentrations (Fig. 7).

3.2.1.2. Thymocytes. Apoptotic index of thymocytes showed a significant increase with increasing dose of corticosterone from 100 ng through 400 ng/ml compared to controls and vehicle controls, whereas, that of 500 ng concentration did not significantly differ from that of 400 ng and was significantly higher than other treatments (Fig. 7).

3.2.1.3. Lymphocytes of axillary lymph nodes. Apoptotic index of lymphocytes of axillary lymph nodes showed significant increase from 100 ng through 500 ng/ml concentrations compared to controls and vehicle controls, whereas, apoptotic index at 100 ng corticosterone concentration did not significantly differ from that of 200 ng (Fig. 7).

4. Discussion

An increase in the adrenocortical activity due to the activation of HPA axis is a familiar response of vertebrates to stressors (Dhabhar, 2014). In the present study, a significant increase in serum concentration of corticosterone coupled with a reduction in body weight of rats following exposure to restraint and forced swimming for different durations indicate that the rats were experiencing stress. Hence, the regressive changes in lymphoid organs were stress responses.

Chronic stress exposure causes immune suppression as shown by the involution of lymphoid organs (Pearse, 2006). The thymus is the primary lymphoid organ required for the generation of mature T lymphocytes (Lee et al., 2016a) and it is most sensitive to adreno-corticol hormones among lymphoid organs (Pearse, 2006). Similar to the earlier reports (Perez-mera et al., 1993; Nyuyki et al., 2012; Lee et al., 2016b), there was a significant reduction in relative weight of thymus in stressed rats compared to controls. However, the present study reveals the shrinkage of cortical region, development of connective tissue and appearance of fat cells in stressed rats. In addition, novel finding is the demonstration that reduction in number of thymocytes is due to increase in apoptosis of thymocytes in stressed rats (*in vivo*) as well as following corticosterone treatment (*in vitro*). Thus, the present study reveals that corticosterone induced apoptotic loss of thymocytes leads to degenerative changes and loss of weight of the thymus in stressed rats.

Spleen is the largest secondary lymphoid organ which defends the body against blood borne pathogens. Splenic macrophages and dendritic cells stimulate the B and T lymphocytes whenever there is microbial infection. Similar to the earlier reports (Perez-mera et al., 1993; Nyuyki et al., 2012; Lee et al., 2016a), there was a significant reduction in relative weight and cellularity of spleen in stressed rats compared to controls. However, present study reveals cause of these changes, *i.e.* this study shows that a significant increase in apoptosis of splenocytes in stressed rats lead to reduction in cellularity and consequently the loss of weight. In addition, the shrinkage of germinal center of white pulp and significant reduction in number of islands of white pulp of the spleen of rats exposed to stressors for three different durations further support damaging effect of stress on spleen.

Lymph nodes are the secondary lymphoid organs which play a major role in defending the body against pathogens and lymphocyte recirculation. Lymphocyte recirculation is a process where in new lymphocytes enter the lymph nodes from blood and if get activated by the pathogens these remain in the lymph nodes, otherwise return back to blood. Few studies reported chronic stress induced reduction in relative weight and lymphocyte population of axillary lymph nodes (Sundaresan et al., 1990; Srinivasan et al., 2016; Sarjan et al., 2017). In

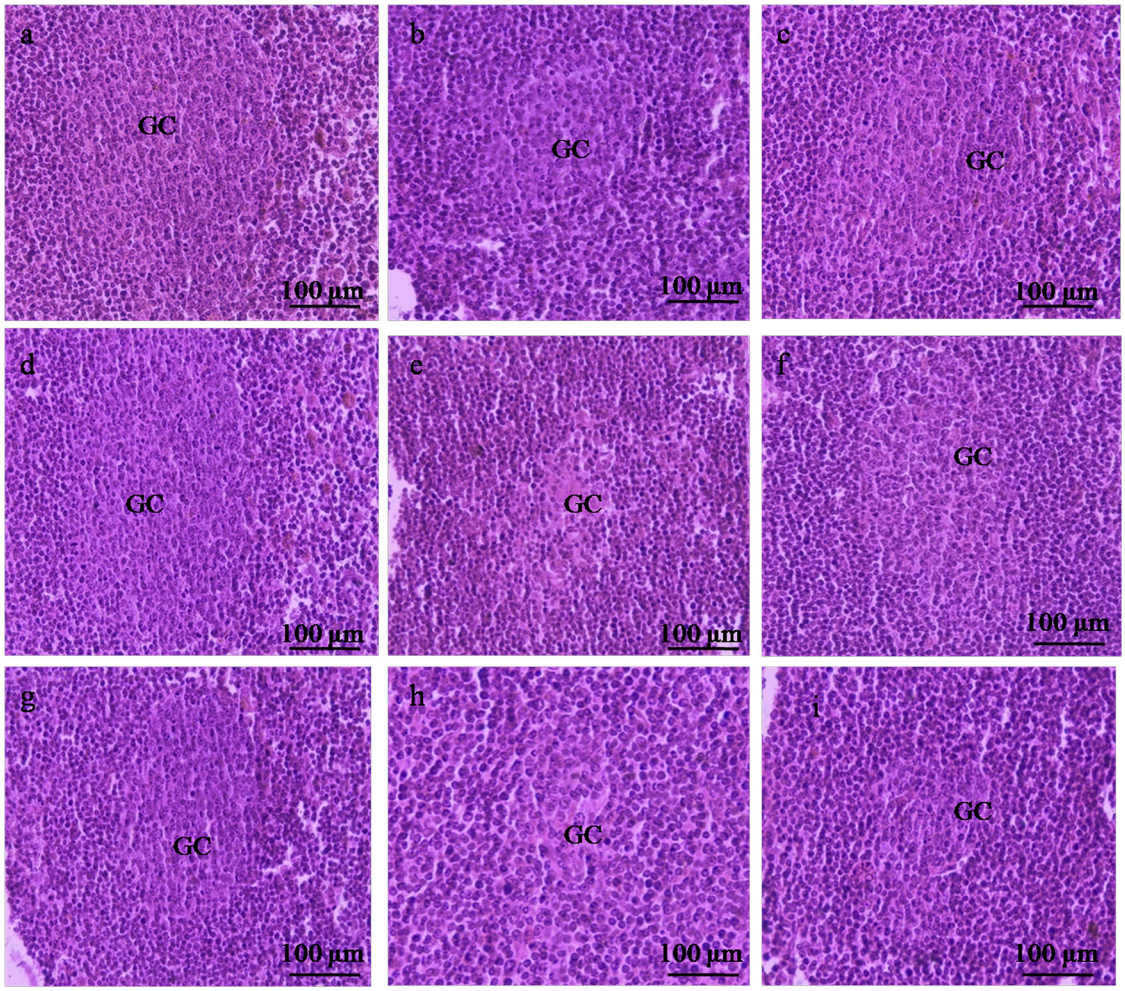

Fig. 6. (a–i) Photomicrographs of cross sections of axillary lymph nodes showing lymphoid follicles. Note the shrinkage of germinal centers in stressed rats of 2 weeks (b), 4 weeks (e) and 8 weeks (h) durations and recovery group rats after 8 weeks stress (i) compared to controls of 2 weeks (a), 4 weeks (d) and 8 weeks (g) durations and recovery group rats after 2 weeks (c) and 4 weeks (f) of stress. 200× (H&E). GC: germinal centre.

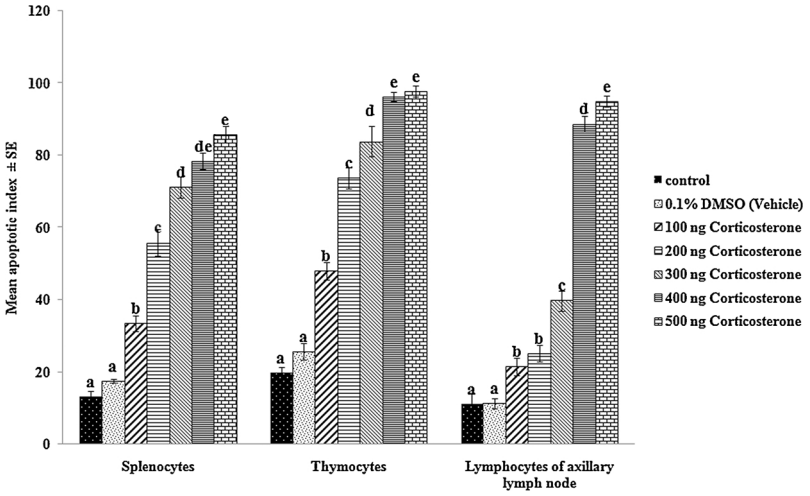

Fig. 7. Vertical bars showing mean apoptotic index of splenocytes, thymocytes and lymphocytes of axillary lymph nodes in different experimental groups following *in vitro* treatment with different doses of corticosterone. Bars with same superscript letters do not significantly differ among themselves whereas those with different superscript letters significantly differ.

the present study, exposure of rats to stressors resulted in a significant increase in the number of apoptotic lymphocytes concomitant with decrease in cellularity and relative weight of axillary lymph nodes. Further, there was shrinkage of lymphoid follicles and germinal centre of lymph nodes. Thus, our study reveals that stress induced apoptotic loss of lymphocytes caused the drastic reduction in population of lymphocytes consequently resulted in the reduction of relative weight of axillary lymph nodes.

It is known that high levels of corticosterone causes apoptosis of immune cells (Schmidt et al., 2004; Marketon and Glaser, 2008; Baschant and Tuckermann, 2010; Zhong et al., 2013). In the present study, stress induced increase in blood corticosterone concentration

was accompanied by apoptosis of cells in spleen, thymus and axillary lymph nodes as shown by florescent staining and there by indicated corticosterone induced apoptosis. This view is further supported by the appearance of DNA ladder in lymphocytes of all lymphoid organs after 8 weeks stress exposure and an increase in apoptosis of lymphocytes of all the lymphoid organs following *in vitro* treatment with corticosterone. The significance of the present study is the demonstration that corticosterone induced apoptosis of lymphocytes of lymphoid organs increases in dose dependent manner and the doses of corticosterone selected are in the *in vivo* range of normal to state of stress, whereas earlier studies (Vicent et al., 2000; Yun-Feng and Zhi-Pu, 2002) have used pharmacological concentrations of corticosterone to demonstrate apoptosis of lymphocytes. The *in vivo* and *in vitro* results put together reveal for the first time that excess of corticosterone secretion due to the stress induced activation of HPA axis causes apoptosis of cells of different lymphoid organs leading to reduction in cellularity and involution of the lymphoid organs.

Earlier studies on effect of stress on lymphoid organs showed significant reduction in the relative weight as well as cellularity of lymphoid organs following short (Morishita et al., 1997; Tarcic et al., 1998) or long term (Dominguez-Gerpe and Rey-mendez, 2001; Zivkovic et al., 2005; Sarjan et al., 2017) exposure. However, it is not clear whether or not the stress effects on lymphoid organs increases with increasing duration of exposure because of a single exposure period in these studies. The facts that, weight of thymus and axillary lymph nodes, lymphocyte counts of spleen, thymus and axillary lymph nodes and number of islets of white pulp of spleen showed duration dependent significant decrease which followed a pattern, control > stress for 2 weeks > 4 weeks > 8 weeks and on the other hand, duration dependent increase in apoptotic index of splenocytes, thymocytes and lymphocytes of axillary lymph nodes followed the pattern, stress for 8 weeks > 4 weeks > 2 weeks > control, clearly indicate that stress effects become more severe with increase in duration of exposure.

Another novel finding is the demonstration of differential stress response of lymphoid organs to chronic stress. All the parameters of lymphoid organs studied showed significant changes in 2 weeks of stress exposure indicating that lymphoid organs are very sensitive to stress effects even in shorter duration of exposure. However, majority of the parameters of thymus showed maximum alterations in 2 weeks of stress exposure and remained in the same level, whereas that of spleen and axillary lymph nodes showed drastic changes after 4 weeks of stress exposure and later remained in the same level. Thus, thymus is more sensitive to stress effects compared to other lymphoid organs. This was further supported by *in vitro* study wherein apoptotic index of thymocytes was higher than that of splenocytes and lymphocytes of axillary lymph nodes with different doses of corticosterone treatment.

It is important to understand whether stress effects on lymphoid organs are reversible or not because long term exposure might cause a severe damage to the organs resulting in irreversible changes. For instance, it is reported that chronic exposure to stress for 6 months caused irreversible damage in testis of rats (Nirupama and Yajurvedi, 2013). Earlier studies on stress and lymphoid organs did not focus on whether stress effects are transient and the alterations revert to normal condition after cessation of stress exposure and if so whether duration of exposure influenced the reversibility of stress effects. Hence, recovery study was conducted wherein rats were allowed to uniform recovery period (6 weeks) after 2, 4 and 8 weeks of exposure. In the recovery group rats after 2 and 4 weeks exposure, all the parameters of spleen and majority of that of thymus and axillary lymph nodes were completely restored to control levels, whereas majority of parameters of these organs of recovery group rats after 8 weeks exposure were similar to stress group, despite 6 weeks of recovery period. Parallely, serum concentration of corticosterone was restored to control levels in recovery groups after 2 or 4 weeks stress but not in that after 8 weeks exposure. A failure of lymphoid organs to return to normalcy following 8 weeks exposure might be due either to continued activation of HPA axis even after withdrawal of stressor after 8 weeks exposure as shown by prevalence of higher levels of serum corticosterone in recovery group rats or irreversible damage to lymphoid organs. It is to be noted that the serum corticosterone concentration in recovery group rats after 8 weeks stress was significantly lower than stress group rats, though it was significantly higher than controls. Therefore, it appears that the serum corticosterone concentration was approaching towards control levels and might reach control levels provided a period longer than 6 weeks to recover. Thus failure to return to control levels during recovery period after 8 weeks exposure appears to be due to higher concentration of corticosterone, even after withdrawal of stressor rather than irreversible damage caused by stress. Further studies involving longer recovery periods are needed to confirm this view. The outcome of this study is revelation that, shorter the stress exposure period, faster is recovery from stress effects.

5. Conclusion

Findings of the present study reveal that increase in duration of stress exposure results in more severe damage to lymphoid organs and that shorter the exposure period, faster the recovery. Further, the study revealed corticosterone induced death of splenocytes, thymocytes and lymphocytes of axillary lymph nodes is the major cause for involution of lymphoid organs.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

Archana, R., 2013. Effect of chronic noise stress on neutrophil functions in rats. AIJRFANS 3, 88–92.
Baschant, U., Tuckermann, J., 2010. The role of the glucocorticoid receptor in inflammation and immunity. J. Steroid Biochem. Mol. Biol. 120, 69–75.
Biase, F.H., Franco, M.M., Goulart, L.R., Antunes, R.C., 2002. Protocol for extraction of genomic DNA from swine solid tissues. Genet. Mol. Biol 25, 313–315.
Dhabhar, F.S., 2014. Effects of stress on immune function: the good, the bad, and the beautiful. Immunol. Res. 58, 193–210.
Divyashree, S., Sarjan, H.N., Yajurvedi, H.N., 2016. Effects of long-term chronic stress on the lymphoid organs andblood leukocytes of the rat (*Rattusnorvegicus*). Can. J. Zool. 94, 137–143.
Dominguez-Gerpe, L., Rey-Mendez, M., 2001. Alterations induced by chronic stress in lymphocyte subsets of blood and primary and secondary immune organs of mice. BMC Immunol. 2, 1–10.
Grissom, N., Kerr, W., Bhatnagar, S., 2008. Struggling behavior during restraint is regulated by stress experience. Behav. Brain Res. 191, 219–226.
Grossmann, J., Maxson, J.M., Whitacre, C.M., Orosz, D.E., Berger, N.A., Fiocchi, C., Levine, A.D., 1998. New isolation technique to study apoptosis in human intestinal epithelial cells. Am. J. Pathol. 153, 53–62.
Hernandez, M.E., Martinez-Mota, L., Salinas, C., Marquez-Velasco, R., Hernandez-Chan, N.G., Morales-Montor, J., Perez-Tapia, M., Streber, M.L., Granados-Camacho, I., Becerril, E., Javier, B., Pavon, L., 2013. Chronic stress induces structural alterations in splenic lymphoid tissue that are associated with changes in corticosterone levels in Wistar–Kyoto rats. Biomed Res. Int. 2013, 1–6.
Hoffman-Goetz, L., Thorne, R., Simpson, J.A., Arumugam, Y., 1989. Exercise stress alters

murine lymphocyte subset distribution in spleen, lymph nodes and thymus. Clin. Exp. Immunol. 76, 307–310.
Lee, Y., Im, S., Kim, J., Lee, S., Kwon, J., Lee, H., Kong, H., Song, Y., Shin, E., Do, S., Lee, C., Kim, K., 2016a. Modified aloe polysaccharide restores chronic stress-induced immunosuppression in mice. Int. J. Mol. Sci. 17, 1–10.
Lee, J.H., Kim, T., Kim, J.W., Yoon, J.S., Kim, H.S., Lee, K., 2016b. The Anti-apoptotic effect of ghrelin on restraint stress induced thymus atrophy in mice. Immune Netw. 16, 242–248.
Marketon, J.I.W., Glaser, R., 2008. Stress hormones and immune function. Cell. Immunol. 252, 16–26.
Moazzam, S., Hussain, M.M., Babar, A., 2013. Response of hypothalamo-pituitary-adrenal axis and immune system to chronic restraint stress in male Sprague dawley rats. Pak. J. Physiol. 9, 29–31.
Morishita, S., Nishi, Y., Sato, E.F., Yamaoka, K., Manabe, M., Inoue, M., 1997. Cold stress induces thymocytes apoptosis in the rat. Pathophysiology 4, 213–219.
Nieto, M.A., Gonzalez, A., Gambon, F., diaz-Espada, F., Lopez-Rivas, A., 1992. Apoptosis in human thymocytes after treatment with glucocorticoids. Clin. Exp. Immunol. 88, 341–344.
Nirupama, M., Yajurvedi, H.N., 2013. Durational effects of chronic stress on the testicular damage and its reversibility in albino rat. Eur. J. Exp. Biol. 3, 229–239.
Nyuyki, K.D., Beiderbeck, D.I., Lukas, M., Neumann, I.D., Reber, S.O., 2012. Chronic subordinate colony housing (CSC) as a model of chronic psychosocial stress in male rats. PLoS One 7, 1–11.
Padgett, D.A., Glaser, R., 2003. How stress influences the immune response. Trends Immunol. 24, 444–448.
Parham, P., 2014. Elements of the immune system and their roles in defense. The Immune System, fourth edition. Garland Science, Taylor and Francis Group, pp. 17–22.
Pearse, G., 2006. Histopathology of the thymus. Toxicol. Pathol. 34, 515–547.
Perez-Mera, M.L., Guerra-Pestonit, B., Rey-Méndez, M., 1993. Thymic response of C57BL/6 mice to three different stressors. Nova Acta Cient. Compostel. 4, 173–177.
Pronin, A.V., Nikolaeva, T.N., Deyeva, A.V., Zaytseva, L.G., Vasilleva, E.I., Kirillicheva, G.B., Baturina, I.G., Soloveva, M.S., Zakharov, V.M., 1997. Social stress in laboratory rats *Rattusnorvegicus* results in decreased immune competence of the offspring. Acta Theriol. (Suppl. 4), 33–40.
Ruddle, N.H., Akirav, E.M., 2009. Secondary lymphoid organs: responding to genetic and environmental cues in ontogeny and the immune response. J. Immunol. 183, 2205–2212.
Sabioncello, A., Gotovac, K., Vidovic, A., Gagro, A., Markotic, A., Rabatic, A., Dekaris, D., 2004. The immune system under stress. Period. Biol. 106, 317–323.
Sarjan, H.N., Divyashree, S., Yajurvedi, H.N., 2017. The protective effect of the Vacha rhizome extract on chronic stress induced immunodeficiency in rats. Pharma Biol. 55, 1358–1367.
Schmidt, S., Rainer, J., Ploner, C., Presul, E., Riml, S., Kofler, R., 2004. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ. 1, 45–55.
Srinivasan, S., Loganathan, S., Wankhar, W., Rathinasamy, S., Rajan, R., 2016. Stress effect on humoral and cell mediated immune response: indispensable part of corticosterone and cytokine in neutrophil function. Trials Vaccinol. 5, 61–70.
Stefanski, V., Peschel, A., Reber, S., 2003. Social stress affects migration of blood T cells into lymphoid organs. J. Neuroimmunol. 138, 17–24.
Sundaresan, G., Sutiianthirarajan, N., Namasnayam, A., 1990. Certain immunological parameters in sub-acute cold stress. Ind. J. Physiol. Pharmacol. 34, 57–60.
Tarcic, N., Ovadia, H., Weiss, D.W., Weidenfeld, J., 1998. Restraint stress-induced thymic involution and cell apoptosis are dependent on endogenous glucocorticoids. J. Neuroimmunol. 82, 40–46.
Tatlican, S., Gulbahar, O., Eren, C., Eskioglu, F., Ergun, M.A., Yilmaz, A., 2009. Apoptotic view to what happens at periphery in psoriasis. J. Turk. Acad. Dermatol. 3, 1–5.
Tournier, J.N., Mathieu, J., Mailfert, Y., Multon, E., Drouet, C., Jouan, A., Drouet, E., 2001. Chronic restraint stress induces severe disruption of the T-cell specific response to tetanus toxin vaccine. Immunology 102, 87–93.
Vicent, G.P., Lanuza, G.M., Rornero, D.M., Croce, L.D., Pecci, A., 2000. Glucocorticoid-induced apoptosis: a simple set of laboratory experiments. Biochem. Mol. Biol. Educ. 28, 307–312.
Yun-Feng, L., Zhi-Pu, L., 2002. Desipramine antagonized corticosterone induced apoptosis in cultured PC12 cells. Acta Pharmacol. Sin. 23, 311–314.
Zager, A., Andersen, M.L., Ruiz, F.S., Antunes, I.B., Tufik, S., 2007. Effects of acute and chronic sleep loss on immune modulation of rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, 504–509.
Zhong, H., Wang, H., Yang, C., Zhou, J., Jiang, J., 2013. Low concentration of corticosterone exert stimulatory effect on macrophage function in a manner dependent on glucocorticoid receptors. Int. J. Endocrinol. 2013, 1–8.
Zivkovic, I., Rakin, A., Petrovic-Djergovic, D., Miljkovic, B., Micic, M., 2005. The effects of chronic stress on thymus innervation in the adult rat. Acta Histochem. 106, 449–458.