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Original Article	**Role of Estrogen Supplementation in Boosting the Immune Response and Stress Resistance as Seen in the White Pulp of the Senile Female Albino Rat Spleen**

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ABSTRACT

Background: Living organisms face various kinds of environmental stressors every day that affect the immune system. The spleen plays key role in the immune responses. There is increasing interest in the immunological role played by estrogen in females especially in cases of stress and senility.

Aim of the work: To study the effect of estrogen administration on the white pulp of the spleen of senile female albino rats and its possible protective role in case of stress exposure on the immune cells, estrogen receptors distribution and cell apoptosis within the white pulp of the spleen.

Materials and Methods: Twenty senile female albino rats were divided into four groups (5 rats/group): Group A, negative control that received daily subcutaneous injection (s.c. inj.) of sesame oil for one week, Group B, rats received s.c. estradiol inj. (40µg/kg BW) for one week, Group C, rats were subjected to immobilization stress for three successive days after s.c. sesame oil inj. for one week, Group D, rats received s.c. estradiol inj.one week before the same stress exposure. All rats were sacrificed at the end of the experiment. Spleen samples were processed for light and electron microscopic examination. Paraffin embedded tissues were sectioned and immune stained in the four groups for estrogen receptors detection, CD3 (T lymphocyte immune marker) and CD20 (B lymphocytes immune marker) distribution also immune-staining for apoptosis was performed in the groups subjected to stress.

Results: Estrogen administration resulted in immune cell proliferation in lymphoid follicles of the spleen; an increase in both CD3 and CD20 positively stained cells and estrogen receptors**.** Stress exposure (Group C) resulted in depletion of the splenic lymphoid follicles, weak immune staining for Estrogen Receptors (ER), dense immune staining for apoptosis, weak immune staining for CD3 and CD20 in contrast to the group protected by estrogen (Group D) which showed preserved white pulp follicles, weak staining for apoptosis marker, preserved immune staining for CD3 and CD20 and positive staining for ER. Electron microscopic examination showed the ultrastructure of lymphocytes in Group A, increased plasma cell in Group B, destructed lymphocytes in Group C and preserved lymphocytes in Group D.

Conclusion: Estrogen administration showed immune cell stimulation in senile rats. In addition, estrogen injection prior to stress exposure seemed to reduce the hazardous effect of stress on the immune cells of the spleen. Exogenous estrogen treatment may become clinically important to enhance the immune response in senile female patients especially in stress exposure.

Key Words: Senile, female rat, spleen, Stress, estradiol, protection, estrogen receptors, CD3, CD20, apoptosis, electron microscopy.

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INTRODUCTION

It is well-documented that post-menopausal decline in the levels of gonadal steroid hormones, including estrogen, could be responsible for the observed elevation in pro-inflammatory cytokines and the decrease in T and B lymphocytes that could promote systemic inflammation seen in the elderly. Recent studies suggested that in addition to age, in postmenopausal women, changes of the immune system have been attributed to estrogen deprivation *(Gameiro & Romão, 2010)*. Hormone replacement therapy (HRT) especially estrogens has multiple influences on immune functions and appears to improve many immune parameters *(Liu et al., 2003; Li & McMurray, 2010)*. Furthermore, recent studies point out to changes in

immune response related to the use or cessation of hormone replacement at menopause *(Gameiro et al., 2010 ; Mathur & Braunstein, 2010).*

An immune-modulatory role for estrogens has been demonstrated by experimental and clinical observations. A variety of mechanisms have been postulated through which estrogen might trigger or exacerbate autoimmunity but the exact mechanism has not yet been clearly defined *(Grimaldi et al., 2002).*

It is generally accepted that chronic stress is suppressive to the immune system. The immune suppression resulting from chronic stress is due in part to a reduction in lymphocyte numbers. Chronic stress can be induced in animals by physical restraint which affects the immune function dramatically *(Wei et al., 2003).* Estrogen was found to prevent oxidative stress that induced apoptosis in rats *(Sudoh et al., 2001).*

The spleen combines the innate and adaptive immune system in a uniquely organized way. The structure of the spleen enables it to remove old erythrocytes from the circulation and leads to efficient removal of blood-borne microorganisms and cellular debris. This function, in combination with a highly organized lymphoid compartment, makes the spleen a very important organ for immune reactivity *(Mebius & Kraal, 2005).*The spleen is also involved in fluid volume regulation, immune responses and hematopoiesis. Recently, it was detected that lymph traversing the spleen acquires specific activation signatures after exposure to the spleen microenvironment *(Semaeva, et al., 2010).*

Living organisms face various kinds of environmental stress every day that affect the immune system. Due to the increasing interest in the immunological role played by estrogen in females and owing to the key role of the spleen in the immune responses, it became the aim of the present work to evaluate the effect and the possible protective role of estrogen on the splenic lymphoid compartment, estrogen receptor distribution, T and B lymphocytes distribution and apoptosis in the spleen of senile female albino rats.

MATERIAL AND METHODS

Chemicals

Folone (Estradiol Benzoate in the form of 5mg/ml oily solution, Misr for Pharmaceuticals Company). The drug was diluted in sesame oil 1:1000 (Misr for Pharmaceuticals Company) and given in a dose of 40µg/kg body weight (10 µg/rat/day) by subcutaneous injection (s.c.) for one week this dose was calculated according to *Suzuki et al. (2006)*. Control rats received daily injections of the vehicle oil (sesame oil) for one week

Animals and grouping

Twenty senile female albino rats (two years old) weighing an average of 275 gm (250-300 gm) were used in the study. Animals were purchased from Research Unit and Bilharzial Research Center of Faculty of Medicine, Ain Shams University. Rats were maintained under routine conditions with free access to food and water, 12 hours light: 12 hours darkness. They were divided into 4 groups (5 animals/ group) as follows:

- **Group A:** Control group where rats received 2ml s.c. sesame oil injection daily for one week.
- **Group B:** Rats received 10 µg estradiol (2ml of the diluted 1:1000 solution in sesame oil) by s.c. injection daily for one week.
- **Group C:** Rats received 2ml s.c. sesame oil injection daily for one week then were subjected to immobilization stress for three successive days.
- **Group D:** Rats received 10 µg estradiol (2ml of the diluted 1:1000 solution in sesame oil) by s.c. injection daily for one week, then were subjected to stress for three successive days.

Induction of stress

Stress effect was induced using the "wrap-restraint" model described by *(Castagliuolo et al. 1996)*. Animals were restrained for 30 minutes by gentle wrapping of their upper and lower limbs with masking tape and then returned to their cages. The stress was repeated for three successive days.

Tissue extraction

Rats in the four groups were sacrificed at the end of the experiments by cervical dislocation. The anterior abdominal wall was opened and the spleen was extracted.

Light microscopy

Specimens were fixed in 10% neutral formalin, dehydrated in ascending grades of ethanol and cleared in xylol. Paraffin blocks were prepared and 5µm sections were cut and stained with haematoxylin and eosin (Hx & E) then examined by the light microscope Vanox light microscope (Olympus, Tokyo, Japan) *(Drury & Wallington, 1980; Bancroft & Gamble, 2002)*.

Transmission electron microscopy

Other specimens from the spleen were cut into 1.0 mm^3 pieces and were immediately dipped in 2.5% glutaraldhyde in phosphate buffer at pH 7.3 and kept in the refrigerator at 4 °C for 2 hrs. After wash with phosphate buffer pH 7.4, they were post-fixed in 1% osmium tetroxide at 4 C for 2 hrs, dehydrated in a graded series of ethanols (50%, 70%, 90 %) and embedded in epoxy resin**.** Semithin sections 1 µm thick were cut using ultramicrotome, stained with toluidine blue, examined and photographed by Olympus 330E microscope. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Under transmission electron microscope (TEM-100 SEO), ultrathin sections were observed and photographed (*Graham & Orenstein, 2007)*.

Immunohistochemistry

Primary Antibodies

1- Rabbit polyclonal anti CD3 antibody (ab5690) with species reactivity to rat, human and mouse (at dilution 1:200) (abcam, pharmaceuticals, USA)

2- Anti-CD20 monoclonal antibody (B9E9) with species reactivity to human and rat (Pierce Biotechnology, Meridian Road Rockford, USA).

3- Anti-Estrogen Receptor polyclonal antibody with species reactivity to rat, human, ovine and was at dilution of 1-2 µg/ml (Pierce Biotechnology, Meridian Road Rockford, USA).

4- Marker for apoptosis; Rabbit Monoclonal Antibody with species reactivity to rat, mouse and human (at dilution 1: 500) (Epitomics, Inc, Mitten Road Suite 103 Burlingame, USA).

Five-micrometer sections were cut from the formalin-fixed, paraffin-embedded blocks; sections were mounted on positively charged glass slides. Sections were first subjected to heat mediated antigen retrieval in sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) prior to blocking in 5% normal goat serum+1% BSA for 2 hours at 37°C. The streptavidin biotin peroxidase method was used for Immune-staining. Spleen sections were deparaffinized in xylene and placed in absolute ethanol. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 20 minutes. To minimize non-specific reaction, sections were initially incubated with fetal calf serum1.5 for 30 min. at 37^0C. Sections were then incubated with the primary antibody overnight at 4^oC and rinsed in phosphate buffer saline (PBS). A biotinylated secondary antibody, diluted 1/100, was applied for 30 min at 37^0C. After another rinse in PBS, sections were incubated with PAP reagent 1:200 for 30 min. at 37^0C .Finally sections were treated with diaminobenzidine-H_2O_2 mixture and counterstained with hematoxylin. Negative control staining was performed after omitting the primary antibody *(Chen et al., 1992; Dabbs et al., 2002)*.

RESULTS:

I. **Light microscopy**

• **Group A (Control senile group):** Examination of Hx&E and toluidine blue-stained sections of the female senile control spleen revealed two distinct zones: White pulp and

red pulp (Fig. 1). The white pulp was subdivided into the Periarteriolar Lymphoid Sheath (PALS), the follicles and the marginal zone (Fig. 1). PALS was composed predominantly of small dark lymphocytes arranged in concentric layers (Figs. 2, 3). The follicles were composed of lymphocytes small and large continuous with the PALS and were found at bifurcation sites of the central arterioles. Some follicles contained germinal centers which appeared paler in colour (Fig. 1). Lymphocytes were characterized by being deeply basophilic cells with scanty cytoplasm and large nucleus (Fig. 2). Some cells stained pale with toluidine blue (Fig. 3). The marginal zone was situated at the interface of the red pulp with the PALS and follicles. It contained less densely packed lymphocytes. A marginal sinus separated the marginal zone from the follicles (Fig. 1).

• **Group B (estrogen-treated):** Splenic sections showed apparent increase in the number of the lymphoid follicles, lymphocytes constituting the follicles and PALS in comparison with the control senile (Group A). Some lymphoid follicles appeared amalgamated together. However, fewer germinal centers were observed than in Group A (Figs. 4-6).

• **Group C (stress group):** This group demonstrated marked lymphocyte depletion of the PALS and follicles in the white pulp as compared with sections of the control senile group (Group A) (Fig. 7). Pale staining of the follicles and around the central arterioles was seen denoting decreased cell numbers (Figs. 7, 8). Most of the lymphocytes surrounding the central arteriole appeared large, pale and were few (Fig. 8).Dilated blood vessels were observed (Fig. 7).

• **Group D (stress+estrogen):** Spleens revealed evident preservation of lymphocytes of PALS and follicles with dark basophilic lymphocytes seen in the white pulp compared to stress group C. Many plasma cells were also detected (Figs. 9, 10).

II. **Estrogen Receptor (ER) immunostaining**

Positive immune staining for ER was seen as a brown color inside the nuclei of different cells of the white pulp of the spleen. The number of positively stained cells remarkably increased in group B (estrogen treated) versus group A (control) (Figs. 11, 12). On the other hand, group C (stress group) revealed only few positively stained cells for ER (Fig. 13). Group D (stress+estrogen) showed positively stained cells that were comparable to those of the group B (Fig. 14).

III. **CD3 immunostaining**

Examination of spleen sections of group A (Control senile) showed CD3 positive cells. These appeared as brown spots and were mainly distributed in PALS (T-cell region). Sporadic CD3 positive cells were also identified in the lymphoid follicles and germinal centers (B-cell region). Group B (Estrogen treated) spleen revealed very dense immune staining in the PALS region in contrast to group A (Figs. 15, 16). In the stress group C, a weak reaction to CD3 was noticed in contrast to the protected group D (Stress+estrogen) (Figs. 17, 18).

IV. CD20 immunostaining

Examination of spleen sections of group A immunostained for CD20 showed the brownish coloration of B lymphocytes distributed in the peripheral part of the lymphoid follicles. Spleen sections of group B showed more B lymphocytes as judged by the brownish coloration of these cells (Figs. 19, 20). Sections of the stress group C showed only few positively stained cells (Fig. 21). However, Group D showed slight increase in positively stained cells compared to Group C (Fig. 22).

V. Immunostaining for apoptosis

Immunostaining for apoptosis was performed in the two groups subjected to stress C and D. Positively stained cells for apoptosis marker were seen distributed throughout the splenic tissue. The number of immunostained cells for apoptosis was remarkably lower in Group D (Stress+estrogen) versus the non-protected group C (Figs. 23, 24).

VI. Transmission electron microscopy

Examination of ultrathin sections of the Group A spleen revealed lymphocytes ultrastructure. The lymphocytes had circular or elliptical outline. The nuclei were large euchro-

matic and revealed plenty of fine chromatin with scattered clumps of condensed heterochromatin lining the nuclear membrane. The cytoplasm showed ill-defined organelles (Fig. 25). In Group B, the cytoplasm of the lymphocytes displayed some cellular organelles and many mitochondria (Fig. 26). Plasma cells were frequently seen in group B and showed nuclei with multiple dense areas of heterochromatin (the characteristic cartwheel or clock-face arrangement). The most conspicuous and characteristic feature of these cells was the well-developed rough endoplasmic reticulum channels. The whole of the cytoplasm was taken up by the parallel-concentric arranged cisterns. Some cells revealed dilated cisterns that were filled with flocculent material (Fig. 27).

Cells demonstrating signs of apoptosis were seen particularly in Group C (stress group). Degenerated cells showed nuclear condensation (pyknosis) or fragmentation (karyorehexis) and vacuolated cytoplasm with loss of discernable cell organelles and ill-defined cell membrane (Fig. 28). In Group D (stress+estrogen) the lymphocytes appeared normal with well-defined cell membrane and intact nuclei. The nuclei were rounded, indented, central or eccentric in position. Occasional pseudopodia were also detected (Fig. 29).

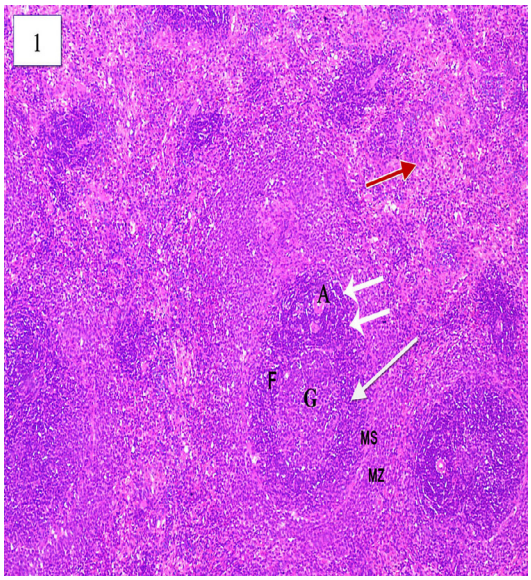

Fig. 1: A photomicrograph of a section of group A (control) spleen showing white pulp (white arrow) and red pulp (red arrow). The white pulp shows periarteriolar lymphoid sheath (PALS, double white arrows), lymphoid follicle (F). Central arteriole (A), germinal centre (G), marginal sinus (MS) and marginal zone (MZ). Hx.&E.; X40

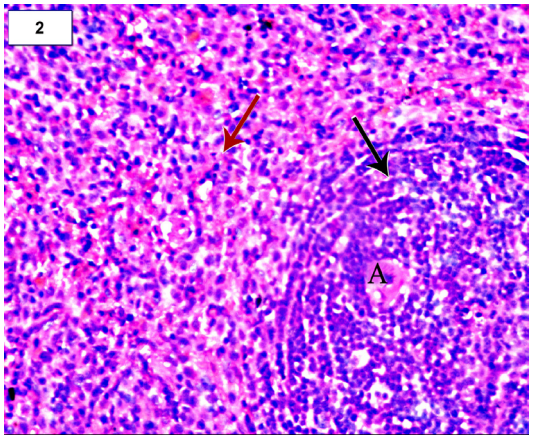

Fig. 2: A photomicrograph of a section of group A spleen showing white pulp formed of basophilic lymphocytic aggregation (black arrow). Central arteriole (A) is seen within the white pulp. The white pulp is surrounded by red pulp (red arrow). Hx.&E.; X100

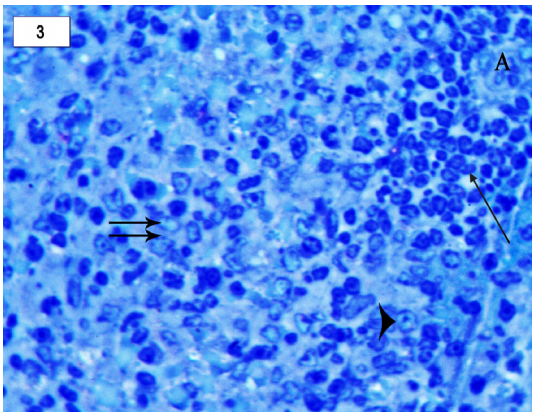

Fig. 3: A photomicrograph of a section of group A spleen showing the PALS formed of predominantly small, dark lymphocytes (arrow) surrounding central arteriole (A). Plasma cells are also seen showing the characteristic cart wheel appearance of their nuclei (arrow head). Other pale staining cells can be seen (double arrows). Toluidine blue; X1000

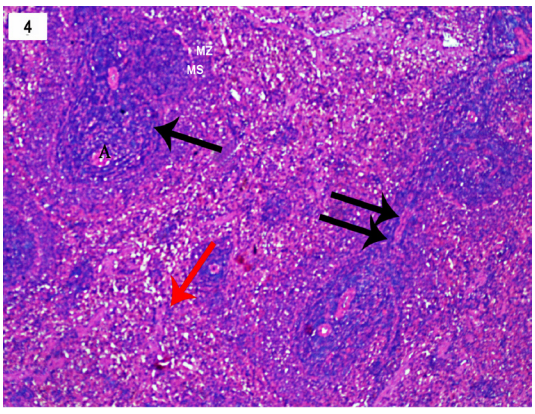

Fig. 4: A photomicrograph of a section of group B spleen showing marked lymphocytic hyperplasia. Amalgamated follicles (black arrow) or connection between adjacent follicles (double arrow) are seen. Central arteriole (A), red pulp (red arrow), marginal sinus (MS) and marginal zone (MZ). Hx.&E.; X40

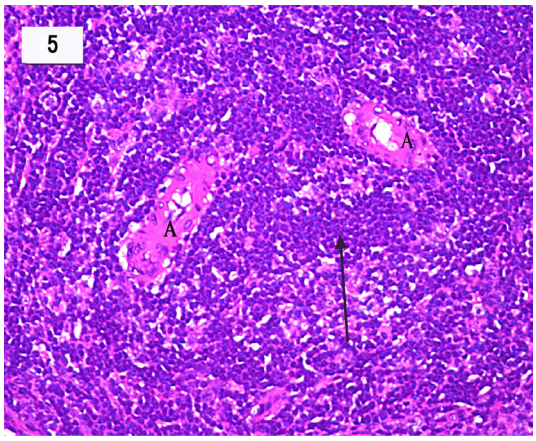

Fig. 5: A photomicrograph of a section of group B (estrogen treated) spleen showing marked proliferation of lymphocytes in the PALS (black arrow) surrounding two central arterioles (A). Hx.&E.; X100

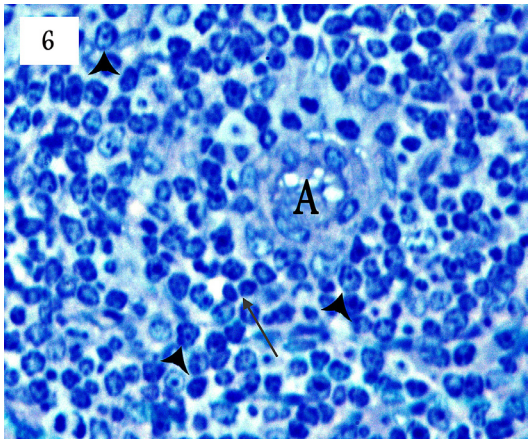

Fig. 6: A photomicrograph of a section of group B spleen showing hyperplasia of lymphocytes (arrow) within the white pulp surrounding central arterioles (A). Many cells with nuclear features of plasma cells can be seen (arrowhead). Toluidine blue; X 1000

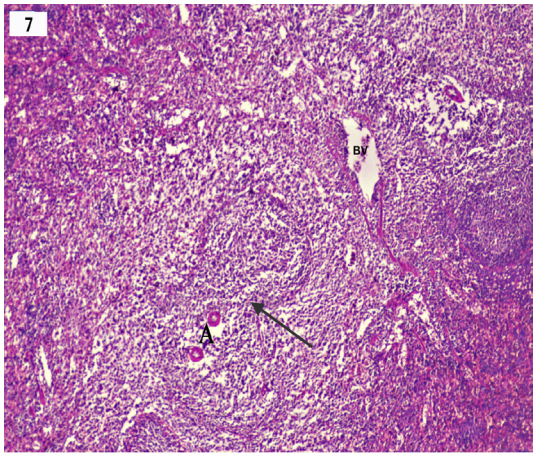

Fig. 7: A photomicrograph of a section of group C (stress group) spleen showing marked white pulp depletion (arrow). Notice the dilated blood vessels (BV) and the central arteriole (A). H.&E.; X40

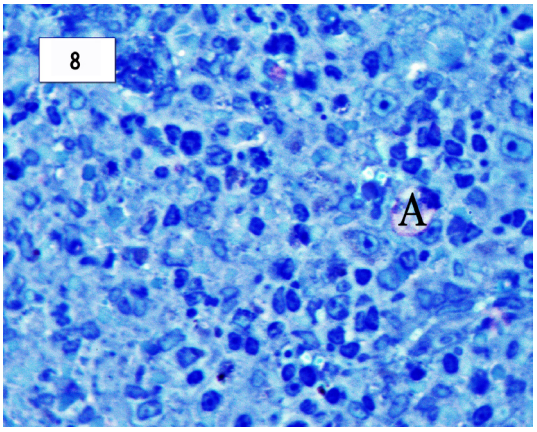

Fig. 8: A photomicrograph of a section of group C (stress group) spleen showing few mostly pale large lymphocytes surrounding the central arteriole (A). Toluidine blue; X 1000

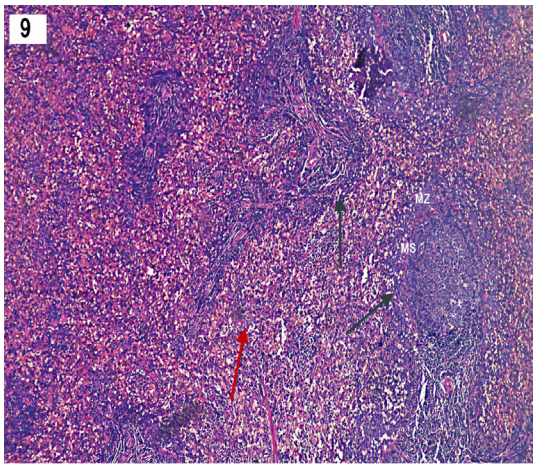

Fig. 9: A photomicrograph of a section of **group D** (stress+ estrogen) spleen showing preserved lymphoid follicles (black arrow) among the red pulp (red arrows).Note the marginal sinus (MS) and the marginal zone (MZ). Hx.&E.; X40

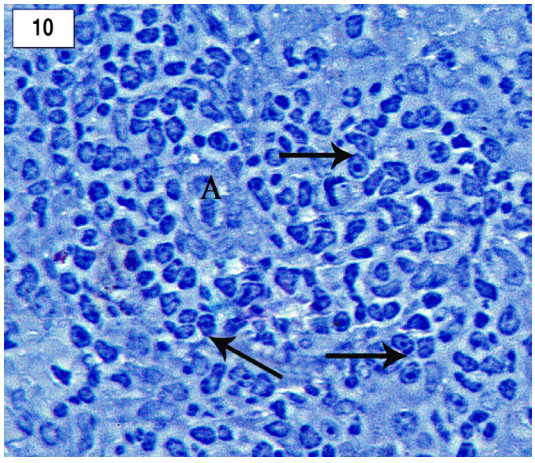

Fig. 10: A photomicrograph of a section of **group D** spleen showing high magnification of the white pulp that revealed preserved lymphocytes surrounding the central arteriole (A). Note the presence of many plasma cells (arrow). Toluidine blue; X 1000

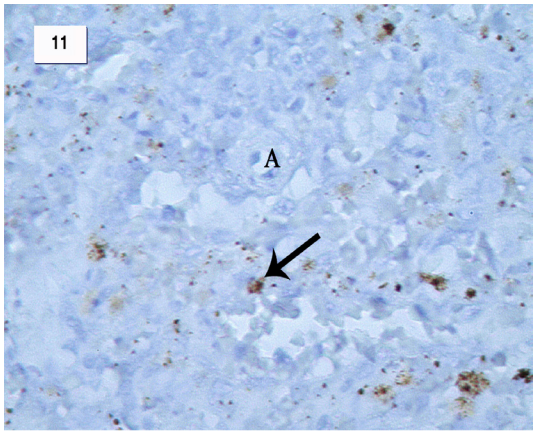

Fig. 11: A photomicrograph of a section of group A (control senile) spleen showing scattered brown color through the section revealing positive immune staining for estrogen receptor (arrow). Note the central arteriole (A). Anti-estrogen receptor antibody; X 200

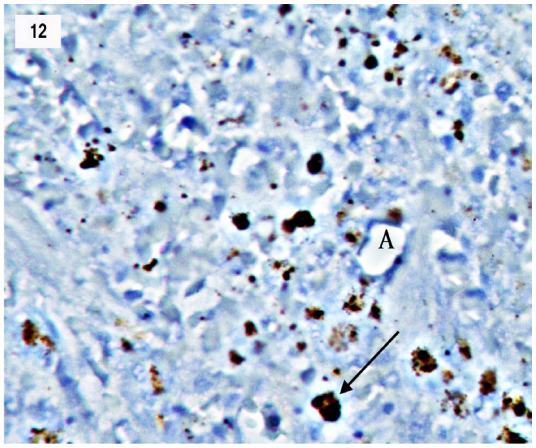

Fig. 12: A photomicrograph of a section of group B (estrogen treated) spleen showing strong positive nuclear staining for estrogenic receptor (arrow). Note the arteriole (A). Anti-estrogen receptor antibody; X 200

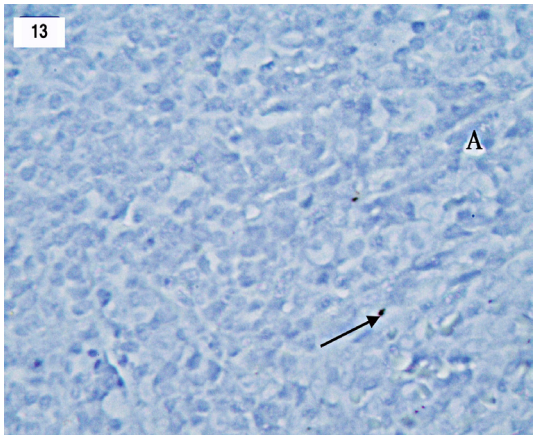

Fig. 13: A photomicrograph of a section of group C (stress) spleen showing very few cells immune stained for estrogen receptor antibody (arrow). Note the arteriole (A). Anti-estrogen receptor antibody; X 200

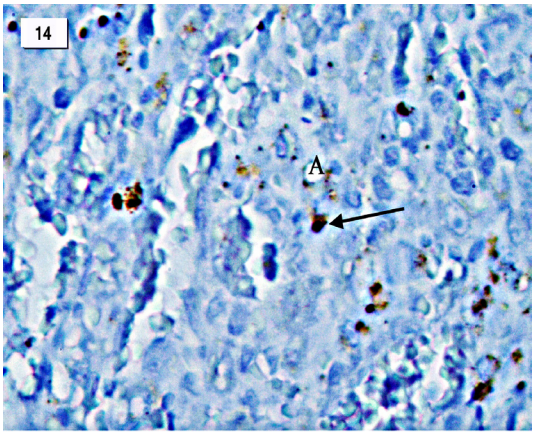

Fig. 14: A photomicrograph of a section of group D (stress+estrogen) spleen showing moderate number of cells immune stained for estrogen receptor antibody (arrow). Note the arteriole (A). Anti-estrogen receptor antibody; X 200

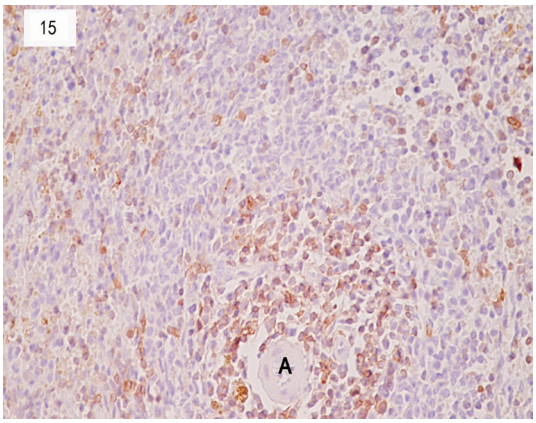

Fig. 15: A photomicrograph of a section of group A (control senile) spleen showing positive immune stained cells (T lymphocytes) for CD3 antibody mainly seen around the central arteriole (A) and revealing brown color. Anti-CD3 antibody; X 100

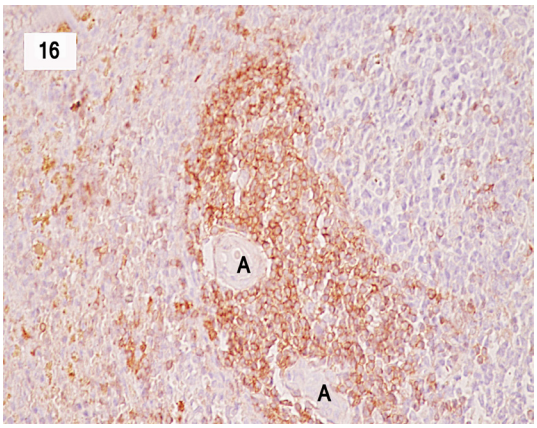

Fig. 16: A photomicrograph of a section of group B (estrogen treated) spleen showing large number of positive immune stained cells for CD3 antibody seen taking the brown color (T lymphocytes) compared to control group A. Immune staining is mainly localized in PALS. Note the central arteriole (A). Anti-CD3 antibody; X 100

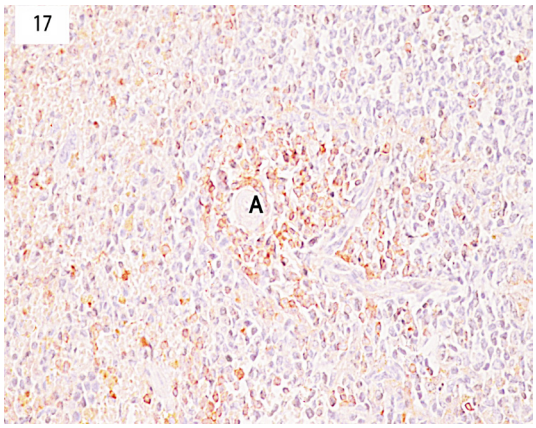

Fig. 17: A photomicrograph of a section of group C (stress) spleen showing few positive immune stained cells (T lymphocytes) for CD3 antibody. Positive cells are mainly seen around the central arteriole (A) and reveal brown color. Anti-CD3 antibody; X 100

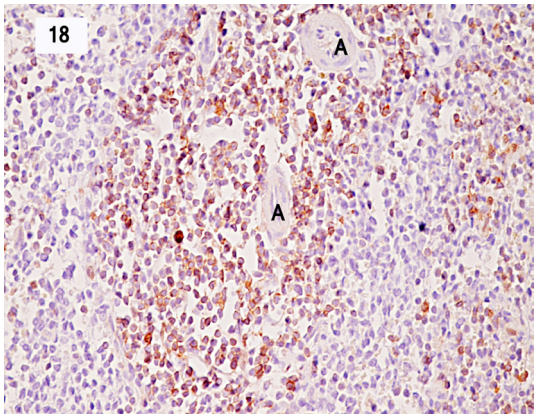

Fig. 18: A photomicrograph of a section of group D (stress+estrogen) spleen showing large number of positive immune stained cells for CD3 antibody taking the brown color (T lymphocytes) compared with group C. Immune staining is mainly localized in PALS. Note the central arteriole (A). Anti-CD3 antibody; X 100

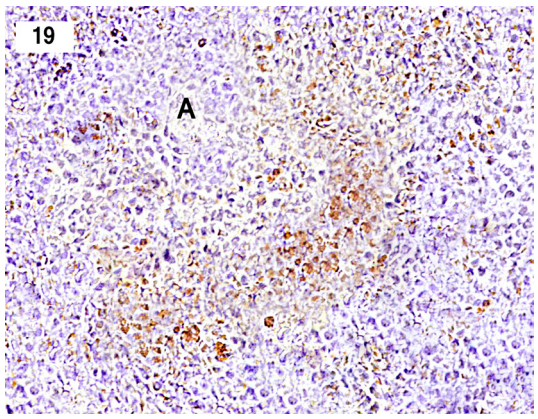

Fig. 19: A photomicrograph of a section of group A (control senile) spleen showing few positive immune stained cells (B lymphocytes) for CD20 antibody. Positive cells are mainly seen at the periphery of the follicles. Note the central arteriole (A). Anti-CD20 antibody; X 100

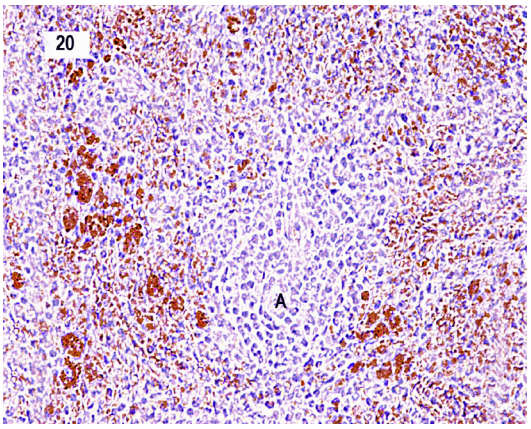

Fig. 20: A photomicrograph of a section of group B (estrogen treated) spleen showing apparent increase in number of positive immune stained cells for CD20 antibody seen taking the brown color (B lymphocytes) compared to group A. Note the central arteriole (A). Anti-CD20 antibody; X 100

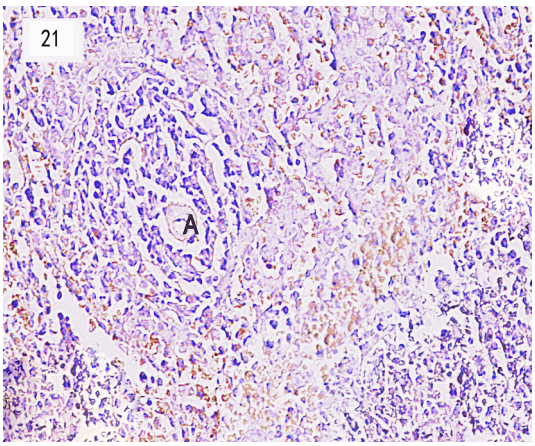

Fig. 21: A photomicrograph of a section of group C (stress) spleen showing few positive immune stained cells (B lymphocytes) for CD20 antibody. Note the central arteriole (A). Anti-CD20 antibody; X 100

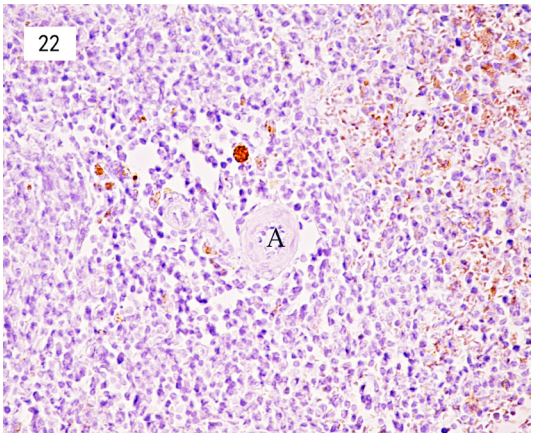

Fig. 22: A photomicrograph of a section of group D (stress+ estrogen) spleen showing slight increase number of positive immune stained cells seen taking the brown color (B lymphocytes) compared with group C. Note the central arteriole (A). Anti-CD20 antibody; X 100

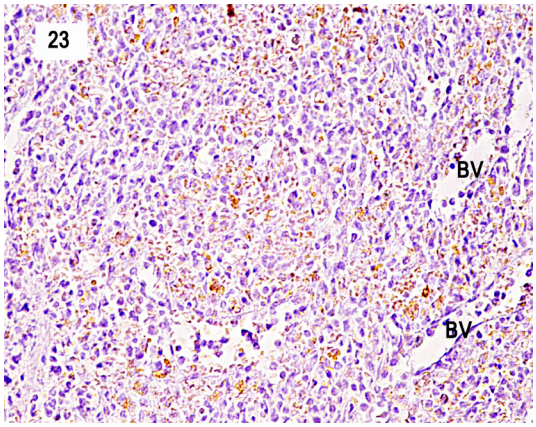

Fig. 23: A photomicrograph of a section of group C (stress) spleen showing evident positive immune stained cells (brown color) for apoptosis antibody. Positive cells are seen distributed throughout the follicle. Notice the dilated blood vessels (BV). Anti-apoptosis antibody; X 100

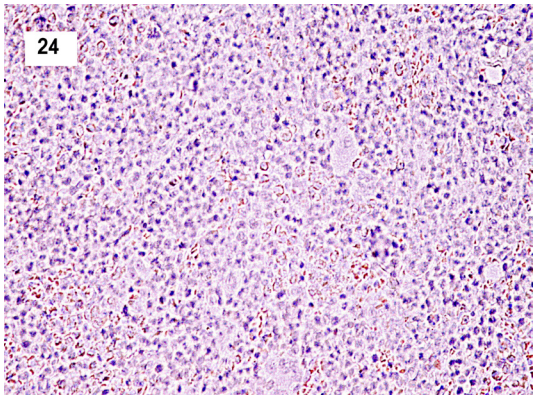

Fig. 24: A photomicrograph of a section of group D (stress+estrogen) spleen showing marked decrease of the number of positive immune stained cells (brown color) for apoptosis antibody compared to group C. Anti-apoptosis antibody; X 100

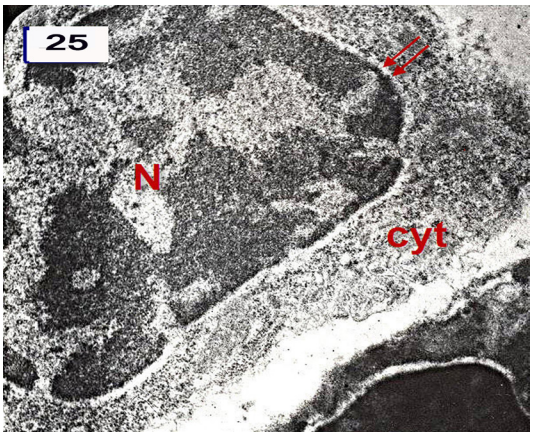

Fig. 25: An electron-micrograph of a section of group A spleen showing a lymphocyte. The nucleus (N) appears euchromatic. The nuclear membrane is lined by clumps of heterochromatin (double arrows). Note the scanty cytoplasm (cyt) surrounding the cell and containing few ill-defined organelles. Uranyl acetate &lead citrate; X10,000

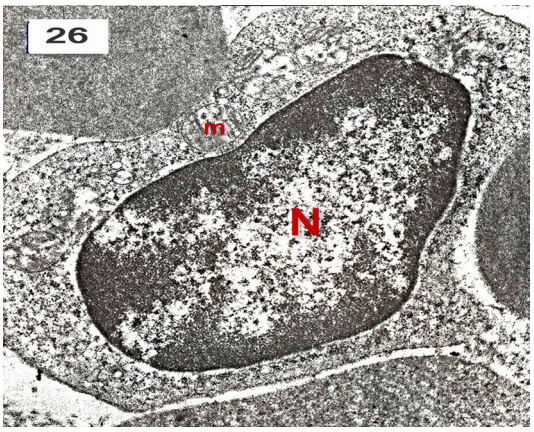

Fig. 26: An electron-micrograph of a section of group B spleen showing a lymphocyte. The nucleus appears euchromatic (N) with heterochromatin lining the nuclear membrane. Numerous mitochondria (m) are seen in the cytoplasm. Uranyl acetate &lead citrate; X 8,000

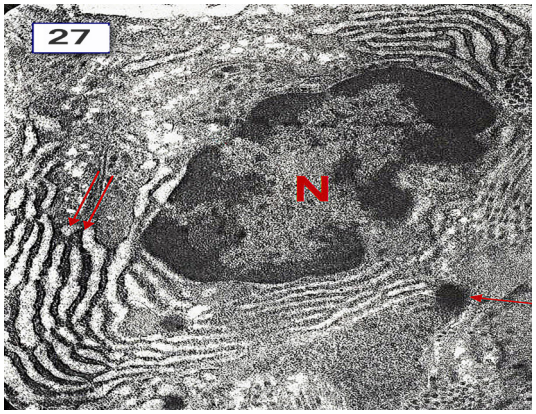

Fig. 27: An electron-micrograph of a section of group B spleen showing a plasma cell. The nucleus (N) shows the characteristic cart wheel appearance. The cytoplasm is totally occupied by the concentric arrangement of rough endoplasmic reticulum channels (double arrows). Secretion products are seen in some channels (arrow). Uranyl acetate &lead citrate; X 9,000

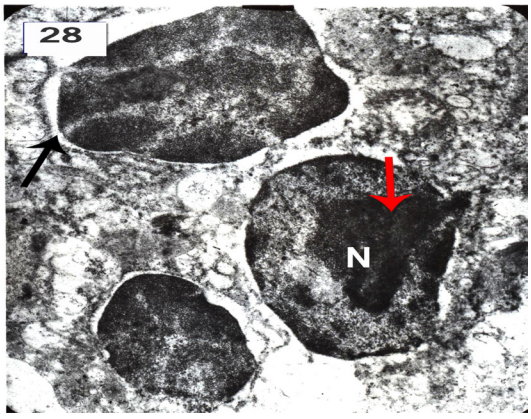

Fig. 28: An electron-micrograph of a section of group C spleen showing lymphocytes revealing signs of apoptosis. The nuclei (N) appear pyknotic (red arrow) and the cytoplasm reveals in-discernable organelles with ill-defined cell membranes. Uranyl acetate &lead citrate; X 6,000

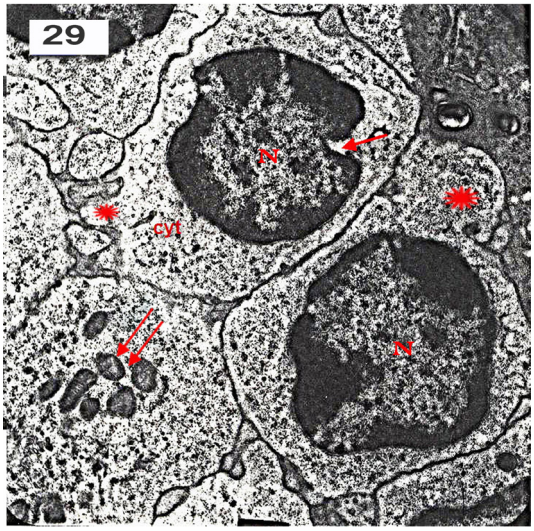

Fig. 29: An electron-micrograph of a section of group D spleen showing lymphocytes. Their nuclei (N) show the normal peripheral location of heterochromatin. The cytoplasm (cyt) is poor in cell organelles with free ribosomes and mitochondria (double arrows).Cell membranes are well defined. Occasional pseudopodia are detected (asterics). Notice the presence of nuclear indentation (arrow). Uranyl acetate &lead citrate; X 6,000

DISCUSSION:

The present work studied the structure of the white pulp of the spleen in female rats under four conditions: Senile rats, senile rats with estrogen supplementation, stress exposure of the senile rats and stress exposure with prior estrogen supplementation.

Sections of the spleen of the control senile rats (Group A) showed the classic structure of the spleen with white pulp and red pulp. The white pulp of the spleen showed the PALS which was formed predominantly of small dark lymphocytes. These cells proved by immunohistochemistry to be CD3 positive or T lymphocytes *(Zhou et al., 2011)*. The CD3 is a protein complex bound to the membranes of all mature T cells and in virtually no other cell type. This high specificity combined with the presence of CD3 at all stages of T cell development makes it a useful immune-histochemical marker for T cell in tissue sections (*Lee et al., 2011*).

CD20 or B-lymphocyte antigen is expressed on all stages of B cell development except the first and last (late pro B cell, plasmablasts and plasma cells respectively) *(Tedder et al., 1988; Gomez et al., 2010)*. In the control senile group, CD20 immunostaining was detected in the peripheral zone of the lymphoid follicles. Other cells in the lymphoid follicles appeared pale and large. These latter cells could be immature lymphocytes, dendritic cells, plasma cells or macrophages. *Elmore (2006)* reported that immature B cells or immunoblasts are larger and paler staining than the mature lymphocytes. These cells would mature to plasma cells and migrate into the red pulp.

In different spleen sections, the marginal sinus was observed separating the marginal zone from the lymphoid follicle of the PALS. *Mebius and Kraal (2005)* described the endothelium of the marginal sinus towards the marginal zone to be leaky permitting access of antigens and recirculating lymphocytes to the marginal zone. They considered the marginal zone to be a separate compartment rather than part of the white pulp. They claimed that it was designed to screen the systemic circulation for antigens and pathogens and play an important role in antigen processing.

In this study, to elucidate relevance of estrogens to immune responses, the presence of estrogen receptors in the rat's splenic B cells was investigated. Estrogen receptors were identified in the cells of rat's splenic white pulp. *Sakazaki et al. (2002*) found estrogen receptors alpha in mouse splenic lymphocytes and correlated this with a role of estrogen in immunity.

Reviewing the literature, there were contradictory reports regarding the effect of estrogen on the immune system. In estrogen-treated rats (Group B), marked immune stimulation was manifested by proliferation of the lymphocytes in the white pulp as noted in Hx&E and toluidine blue stained sections. This observation was further documented by immune staining for CD3 and CD20 that revealed marked increase in the number of positively stained cells. The present findings are in accordance with previous reports on the immune stimulatory effect of estrogen administration *(Bird et al., 2008; Cunningham & Gilkeson, 2011)*. *Cesta (2006)* commented that aging could decrease lymphocytes numbers by 80%. Estrogen administration to senile rats in this work caused a boosting in the immune system by the increase in lymphocytes. *Nalbandian and Kovats (2005)* reported that high pharmacological doses or pregnancy levels of estrogen alter and perhaps inhibit cell

mediated immunity. *However*, *Li and McMurray (2010)* as well as *Gameiro and Romão (2010)* found that low doses of estrogen improved many immune parameters. Thus according to the estrogen dose, whether high or low, the response may be inhibitory or stimulatory to the immune system.

In the present work, sections from spleens of group B showed more plasma cells compared to Group A. *Cesta (2006)* reported that plasma cells are the end stage of B cell activation. Plasma cells were recognized by EM by their well-developed rough endoplasmic reticulum.

Stress can be defined as physical and psychological modifications that disrupt the homeostasis and the balance of organisms. Stress is known as one of the most important reasons of several diseases and is associated with immune dysregulation *(Bakhmet, 2004; Wierzba et al., 2006; Guéguinou et al., 2011)*. In the present study, the spleen of the rats exposed to stress (Group C) revealed decrease in lymphocyte numbers as demonstrated by depletion of the white pulp in Hx&E & toluidine blue-stained sections. On the contrary, the spleen in the group that received estrogen prior to stress (Group D) showed salient preservation of the lymphoid compartment of the white pulp. Signs of cell destruction seen by electron microscopy in Group C in the present study were much prevented in the protected group D. Moreover, in group D lymphocytes showed pseudopodia. These pseudopodia represent one of the cell defensive mechanisms in response to antigens *(Anvari et al., 2004)*.

Immune staining for ER showed marked decrease in the number of positively stained cells in group C in contrast to group D. This adds further evidence for the protection afforded by estrogen to immune cells from damage caused by stress exposure. These results are in accordance with previous investigations on the stimulatory effect of estrogen on immune cells *(Sármay et al., 2010)* and on the ability of estrogen to modulate the differentiation of hematopoietic cells via the action on the uncommitted lymphoid and myeloid progenitors in the bone marrow (*Nalbandian & Kovats, 2005*).

Further supporting evidence for lymphocyte distribution in groups C and D was studied by immune staining for T and B lymphocytes using CD3 and CD20 markers respectively. Using anti-CD3 antibody, immune staining demonstrated marked reduction in the number of T cells in the stress-exposed group (Group C) compared to the estrogen protected group (Group D). Thus, Estrogen administered prior to stress exposure resulted in T cell preservation. The present data is in accordance with previous reports *(Bird et al., 2008; Sármay et al., 2010)*. Immune staining with anti-CD20 antibody for groups C and D revealed weak reaction in the two groups with more negative reaction in group C. Stress per se results in humoral immune response which implies the activation and differentiation of B lymphocytes into the antibody producing plasma cell with resultant decrease in the number of resting B cells *(Verthelyi & Ahmed, 1998; Bynoe et al., 2000; Guéguinou et al., 2011)*. In addition, estrogen administration induces activation of normal murine B cells and the production of autoantibodies *(Ansar Ahmed et al., 1989; Lagerquist et al., 2008)*. Taking all this together and considering that CD20 is a marker for mature but not activated B cells could explain the weak immune staining for CD20 antibody in groups C and D in the present work.

In the present study, dilated blood vessels were seen in the stressed non-protected spleen (Group C). Previous studies demonstrated that after stress splenic vasoconstriction occurs followed by vasodilatation. Immune processes cause only a local increase in blood flow which would favor splenic cell uptake of antigens and immune defense. On the contrary, general vasodilatation would interfere with the capacity of the spleen to extract cells from the circulation and thus interfere with splenic immune functions *(Rogausch et al., 2003)*.

Although estrogen is largely recognized as a reproductive hormone, it can also protect various cell types during acute stress *(Nickerson et al., 2006)*. *Hildebrand et al. (2006***)** added that the depression in cell-mediated immune function following trauma-hemorrhage is shown to be restored by 17ß-estradiol (E2) administration. Estrogen receptors play the predominant role in mediating the beneficial effects of E2 on splenic macrophages and splenocytes. Estrogen exerts its effects on target organs by interacting with specific Estrogen Receptors (ER),

such as ER-and-ß. Splenocytes and splenic macrophages express both of these receptors**.** Current studies have shown that administration of 17β-estradiol prevents trauma-hemorrhage-induced increase in proinflammatory cytokine production and associated multiple organ injury. Moreover, activation of Peroxisome Proliferator-Activated Receptor γ (PPARγ) following ischemic conditions has been shown to be protective, 17β-estradiol administration following trauma-hemorrhage proved to elevate PPARγ activity level *(Suzuki &Kawasaki, 2011)*.

Moreover, reduced risk and severity of stroke in adult females is thought to depend on normal endogenous levels of estrogen; a well-known neuro-protectant and immune-modulator. In male mice, experimental stroke induces immunosuppression of the peripheral immune system characterized by a reduction in spleen size and cell numbers and decreased cytokine and chemokine expression. However, stroke-induced immunosuppression has not been evaluated in female mice. *Zhang et al. (2010)* demonstrated for the first time that E2 replacement in ovariectomized female mice improves stroke-induced peripheral immunosuppression.

Mammalian systems respond to environmental stress by either adapting or undergoing programmed cell death. Apoptosis can be initiated following intrinsic signals that are produced following cellular stress *(Grimaldi et al., 2002; Dash et al., 2003)*. Immune staining for apoptosis revealed strong staining reaction in the stress exposed group (Group C) and is in accordance with the work of *Curtin and Cotter (2003), Liu et al. (2003) and Zhang et al.(2010)*. Electron microscopic observation for group C confirmed the cell death by evident signs of pyknosis, nuclear fragmentations or karyorrhexis and ill-defined cell membrane. However, prior administration of estrogen in group D before stress proved protective as evidenced by less observed apoptosis in immune staining and EM observation.

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دور الاستروجين فى شحذ المناعة و مقاومة الاجهاد وذلك كما يتضح فى تركيب اللب البيض للطحال فى اناث الفئران البيضاء المسنة

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ملخص البحث

مقدمة: تواجه الكائنات الحية العديد من صور الاجهاد البيئي يوميا و التي تؤثر بالتبعية علي الجهاز المناعي و يلعب الطحال دور رئيسي في الاستجابات المناعية. كذلك يوجد اهتمام متزايد بتأثيرات الاستروجين علي الجهاز المناعي في الاناث و خصوصا المسنات وبالذات في حالات الاجهاد.

الهدف من البحث: هو دراسة تأثير تناول عقار الاستروجين علي الخلايا المناعية للب الأبيض للطحال في انثي الفئران البيضاء المسنة والمعرضة للأجهاد.

مواد البحث و اساليب العمل: تم تقسيم عشرين من اناث الفئران البيضاء المسنة الي اربعة مجموعات: المجموعة الاولي و هي المجموعة الضابطة تم حقنها تحت الجلد بزيت السمسم يوميا ولمدة اسبوع و المجموعة الثانية تم حقنها تحت الجلد بعقار الاستراديول يوميا و لمدة اسبوع و المجموعة الثالثة تم حقنها يوميا تحت الجلد بزيت السمسم لمدة أسبوع ثم تعريضها لاجهاد عدم الحركة لمدة ثلاثة أيام متتالية و المجموعة الرابعة تم حقنها بعقار الاستراديول تحت الجلد يوميا لمدة أسبوع ثم تم كذلك تعريضها لاجهاد عدم الحركة لمدة ثلاثة أيام. في نهاية التجربة تم التضحية بالفئران و استخراج عينات الطحال وتمريرها للفحص بالميكروسكوب الضوئي والالكتروني النافذ . كذلك تمت صباغة شرائح البارفين بعدة صبغات مناعية لعدد من الاجسام المضادة مثل مستقبلات الاستروجين ، الخلايا الليمفاوية تي و بي وأيضا موت الخلايا المبرمج .

النتائج: تسبب حقن الاستروجين في المجموعة الثانية في تكاثر عدد الخلايا الليمفاوية في اللب الابيض و زيادة عدد الخلايا المصبوغة مناعيا بمستقبلات الاستروجين مقارنة بالمجموعة الضابطة. أما المجموعة الثالثة التي تعرضت للاجهاد فقد أوضحت نضوب خلايا اللب الابيض و ضعف الصبغة المناعية لمستقبلات الاستروجين و كذلك ضعف الصبغة المناعية للخلايا اليمفاوية تي و بى و زيادة عدد الخلايا المتعرضة للموت المبرمج .أما المجموعة الرابعة فقد أظهرت تحسنا ملحوظا في عدد الخلايا الليمفاوية في اللب الابيض و زيادة عدد الخلايا المصبوغة مناعيا لمستقبلات الاستروجين والخلايا الليمفاوية و نقص في عدد الخلايا التي أظهرت الموت المبرمج وقد أوضحت دراسة الميكروسكوب الالكتروني التركيب الدقيق للخلايا الليمفاوية و خلايا البلازما و الخلايا التي تعرضت للموت المبرمج.

الاستنتاج: حقن عقار الاستروجين أدي الي تنبيه الخلايا المناعية للطحال في انثي الفئران المسنة. كما أدي حقن الاستروجين قبل التعرض للاجهاد الي تخفيض التأثيرات الخطيرة للاجهاد علي الخلايا المناعية للطحال، و من ثم قد يؤدي استخدام عقار الاستروجين في المجال الاكلينيكي الي تحسين الاستجابة المناعية للمرضي و خصوصا الاناث المسنات.