

**PERIPUBERTAL OVARIECTOMY PROVIDES LONG-TERM POSTPONEMENT OF AGE-ASSOCIATED DECLINE IN THYMIC CELLULARITY AND T-CELL OUTPUT**

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The present study was undertaken to reassess the recently challenged role of ovarian hormones in age-associated thymic involution. For this purpose, in eleven-month-old peripubertally ovariectomized (Ox) rats we analyzed: i) thymic weight and cellularity, ii) size of CD4+CD8+ double-positive (DP) thymocyte population, which is believed to correlate to the thymic capacity to export mature T cells, iii) number of recent thymic emigrants (RTEs), and iv) number of peripheral blood CD4+ and CD8+ lymphocytes. It was found that both thymic weight and cellularity were greater in Ox than in control rats. In addition, in Ox rats the numbers of DP thymocytes and both CD4+ and CD8+ RTEs, were significantly greater than in controls, indicating a more efficient generation of T cells in these rats. Furthermore, these findings, coupled with data indicating that the number of neither CD4+ nor CD8+ peripheral blood lymphocytes was affected by ovariectomy, most likely, suggest a reduced homeostatic proliferation of memory cells in Ox rats, i.e. broadening of TCR peripheral repertoire without changes in the overall number of T cells leading to a more efficient response to newly encountered antigens. The results indicate that the ovarian steroid deprivation from early peripubertal period leads to a long lasting postponement/alleviation of age-associated decline in T-cell mediated immune response.

**Key words:** ovariectomy, ageing, thymic cellularity, recent thymic emigrants

**INTRODUCTION**

It is well established that a decline in the immune system function with advancing age termed immunosenescence leads to multiple clinical implications, including increased susceptibility of the elderly to microbial infections (Louria *et al.*, 1993; Pinner *et al.*, 1996; Castle 2000; Schmader, 2001; Aspinall, 2003), increased prevalence of some malignant and autoimmune

diseases (Ershler and Longo, 1997; Pawelec, 1999; Castle, 2000; Burns and Leventhal, 2000; Malaguarnera et al., 2001), delayed immune recovery from immunodeficiency states after cytotoxic antineoplastic treatments and chronic infections and reduced response to vaccinations (Goldberg et al., 2007).

Although consisting of several dynamic immunological alterations the majority of age-related change in the immune functions are ascribed to changes in certain T-cell subset representation and function, which can be attributed to the thymus involution (Makinodan and Kay, 1980; Utsuyama and Hirokawa, 1987; Hirokawa et al., 1994).

Thymic involution is the most dramatic age-associated change in the immune system and it reflects the loss of organ mass and cellularity leading to a decrease in T-cell output, i.e. the number of lymphoid emigrants entering the peripheral T-cell pool (Stutman, 1978; Thoman, 1995; Berzins et al., 2002). This reduction in T-cell output from the thymus initiates the homeostatic expansion of pre-existing memory cells (Ernst et al., 1990; Utsuyama et al., 1992; Kurashima et al., 1995), and is thought to be responsible for narrowing of the TCR repertoire (Mosley et al., 1998; LeMaoult et al., 2000) leading to less efficient responses of elderly to newly encountered antigens (Miller, 2000).

In rodents, immunological functions sharply increase after birth reaching the peak around puberty and gradually decline with advancing age (Grossman, 1985; Hirokawa et al., 1994). The peripubertal rise in the levels of gonadal hormones has been causally linked to the initiation of thymic involution, while post-pubertal high levels of gonadal hormones has been related to the maintenance of thymic involutive changes (Utsuyama et al., 1989; Bodey et al., 1997). This view is supported by numerous studies showing that: i) rise in circulating levels of both ovarian and testicular gonadal steroids, which is induced by the hormone administration, in rodents causes thymic atrophy similar to that seen in ageing (Kuhl et al., 1983; Luster et al., 1984; Windmill et al., 1993; Dulos and Bagchus, 2001; Oner and Ozan, 2002; Yellayi et al., 2002), and ii) surgical gonadectomy before puberty postpones thymic involution, while later in life it produces reversal of ageing-induced changes in the thymus in both sexes (Utsuyama and Hirokawa, 1989; Kendall et al., 1990; Blacker et al., 1991; Windmill et al., 1993; Rao et al., 1996; Leposavić et al., 1996; 1999; Windmill and Lee, 1998; Heng et al., 2005). It should be pointed that in all these studies the effects of gonadectomy have been followed not longer than for 30 days following surgery.

The recent findings obtained by following the thymus cellularity in mice of both sexes and male rats for a longer period of time (Min et al., 2006; Pešić et al., 2007) have challenged the long-time commonly held view that gonadal hormones have a pivotal role in the induction and maintenance of thymic involution. Namely, it has been found that: i) the increase in thymic weight observed in C57BL/6J (B6) mice of both sexes 2 weeks post-gonadectomy performed at the age of 4 weeks is transitory, so that at the age of 24 weeks the thymic cellularity does not differ between gonadectomized and control animals (Min et al., 2006) and ii) at the age of 10 months the thymic cellularity does not differ between male rats castrated at the age of 30 days and their respective controls (Pešić et al., 2007).

The present study was undertaken to reassess the putative role of gonadal hormones in the induction and, particularly, in the maintenance/progression of age-associated thymic atrophy in female rats by examining the effects of long-term ovarian hormone deprivation on thymic cellularity and function, i.e. the thymic T cell output that provides a broadening of the peripheral TCR repertoire from which a high-affinity immune response can be generated. More specifically, in eleven-month-old female rats ovariectomized (Ox) at the age of 30 days we examined: i) the thymic weight and cellularity, ii) the size of double-positive (DP) thymocyte population, which is believed to correlate to the thymic capacity to export mature T cells (Ferrando-Martinez et al., manuscript in press), iii) number of recent thymic emigrants (RTEs) in peripheral blood, and iv) cellularity of CD4+ and CD8+ peripheral blood T-cell compartments.

#### MATERIAL AND METHODS

##### *Animals*

Female Albino Oxford (AO) rats, born and maintained in the animal housing facility at the Immunology Research Centre "Branislav Janković" in Belgrade were selected for the present study. Thirty-day-old animals were subjected to bilateral ovariectomy under pentobarbitone sodium (60 mg/kg, Sagatal, Rhône Mérieux, Ltd., Harlow, UK; i.p.) anaesthesia. After a flank dorsal incision the gland was removed and the incisions were kept closed with metallic clips. For surgical stress control, the same procedure, but without removal of the ovaries, i.e. a sham ovariectomy was performed. Given that there was no statistical difference in any of the examined parameters between sham-ovariectomized and intact rats the data from these two groups were combined. Prior and after the surgery animals were kept in polyethylene cages containing sterilized wood shavings, under controlled conditions of light (12 h light: 12 h darkness, lights on at 07.00 h) and temperature ( $22 \pm 1^\circ\text{C}$ ), and had free access to laboratory chaw and tap water.

The rats were sacrificed ten months after the surgery. Animals showing overt signs of illness, including low body weight, tumors or splenomegaly were excluded from the study. Each experimental group consisted of at least 5 animals.

All procedures involving animals and their care were approved by our Institutional Animal Care and Use Committee and followed principles described in the European Community's Council Directive (86/609/EEC).

##### *Chemicals, antibodies and immunoconjugates*

Sodium azide was purchased from Sigma-Aldrich, (Taufkirchen, Germany). Fetal calf serum (FCS) was purchased from Gibco, Grand Island, NY, USA.

For immunphenotyping the following monoclonal antibodies (mAbs) were used: phycoerythrin (PE)-conjugated anti-CD45RC (clone OX-22), fluorescein-isothiocyanate (FITC)/PE-conjugated anti-CD4 (clone OX-38), FITC-conjugated anti-CD8 (clone OX-8), peridinin chlorophyll protein (PerCP)-conjugated anti-CD90 (Thy-1.1) (clone OX-7), purchased from BD Biosciences Pharmingen (Mountain View, CA, USA).

*Tissue and blood collection and single-cell preparation*

Each thymus was aseptically isolated, trimmed of fat and connective tissue, gently blotted on gauze to remove excess blood and weighted. A single-cell suspension of thymocytes was prepared by forcing the tissue through a 60- $\mu\text{m}$  sieve screen using the rubber end of a syringe plunger into ice-cold phosphate-buffered saline (PBS) pH 7.3 containing 2% FCS and 0.01% sodium azide (FACS buffer).

Blood samples were taken by cardiac puncture and hemolyzed by the addition of ammonium chloride solution (warmed to room temperature) to blood samples in a volume ratio of 1:5.

The resulting cell suspensions were washed three times in ice-cold FACS buffer. After three washes in FACS buffer, single-cell suspensions were counted in an improved Neubauer haemocytometer and cell density was adjusted to  $1 \times 10^7$  cells/mL by addition of FACS buffer. The viability of such cell preparations (as determined by Trypan blue exclusion) was routinely greater than 95%.

*Flow cytometric staining and analysis*

Aliquots of  $1 \times 10^6$  lymphoid cells in 100  $\mu\text{L}$  of FACS buffer were centrifuged at  $350 \times g$  for 5 min at 4°C to yield a pellet. The pellet was subsequently resuspended in FACS buffer and the cell suspensions were incubated for 30 min at 4°C in the dark with FITC-, PE-, or PerCP-conjugated mAbs and then washed twice in FACS buffer.

All samples were analyzed on the same day using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Non-specific IgG isotype-matched controls were used for each fluorochrome type to define background staining, while dead cells and debris were excluded from analysis by selective gating based on anterior and right-angle scatter. The percentage of positive cells for each staining was determined using CellQuest software (Becton Dickinson).

*Statistical analysis*

Statistical comparisons between groups were performed using the Mann-Whitney U test, with a statistical package SPSS for Windows 10.0. Differences in values at  $P < 0.05$  were considered to be significant.

RESULTS

*Ovariectomy at peripubertal age evoked long-lasting increase  
in the thymic weight and cellularity*

In eleven-month-old rats Ox peripubertally, the thymic weight and cellularity were significantly ( $P < 0.01$ ) increased over the corresponding values in age- and sex-matched control rats (Table 1), so that in ovariectomized rats the average thymic weight was approx. 2.5 time greater, while the average thymic cellularity was approx. 2.7 time greater compared to the corresponding values in control animals. Neither the increase in thymic weight nor the rise in the thymic cellularity

was likely to reflect changes in body weight since both the relative thymic weight, i.e. thymic weight per 100 g body weight (median, 25<sup>th</sup> percentile-75<sup>th</sup> percentile) (0.12 g/100 g BW, 0.10 g/100 g BW – 0.16 g/100 g BW vs 0.07 g/100 g BW, 0.06 g/100 g BW – 0.08 g/100 g BW in controls) and relative thymocyte number, i.e. number of thymocytes per 100 g body weight (1.06 x 10<sup>8</sup>/100 g BW, 0.89 x 10<sup>8</sup>/100 g BW – 1.35 x 10<sup>8</sup>/100 g BW vs 0.53 x 10<sup>8</sup>/100 g BW, 0.50 x 10<sup>8</sup>/100 g BW – 0.62 x 10<sup>8</sup>/100 g BW in controls), were significantly ( $P<0.01$ ) greater in peripubertally Ox rats compared to age- and sex-matched controls.

Table 1. Peripubertal ovariectomy induced increase in the thymic weight and cellularity in eleven-month old rats thymic compared with age- and sex-matched control rats

Treatment	Thymic weight (g)	Total number of thymocytes ( $\times 10^8$ )/thymus
	median (25 <sup>th</sup> -75 <sup>th</sup> percentiles)	median (25 <sup>th</sup> -75 <sup>th</sup> percentiles)
Control group (n=11)	0.13 (0.12-0.16)	1.02 (0.94-1.25)
Ox group (n=5)	0.34 (0.30-0.38)**	2.89 (2.54-3.27)**

\*\* $P<0.01$

*Ovariectomy at peripubertal age produced long-lasting increase in the percentage and the absolute number of CD4+CD8+ double positive thymocytes*

As a thymic function surrogated marker (Ferrando-Martinez et al., manuscript in press), the absolute number of CD4+CD8+ double positive (DP) thymocytes was estimated in both Ox and control rats. Significant rise in both the

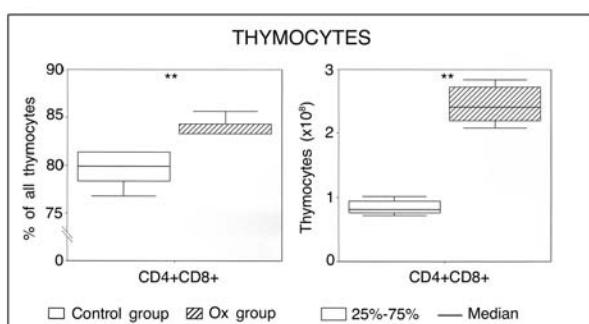


Figure 1. Peripubertal ovariectomy increased the percentage and the absolute number of CD4+CD8+ double positive thymocytes in eleven-month-old rats.

Box plots represent (left) relative proportion and (right) numbers of CD4+CD8+ double positive thymocytes in peripubertally ovariectomized (Ox group) and age-matched control rats (Control group). \*\* $P<0.01$

percentage ( $P<0.01$ ) and absolute number ( $P<0.01$ ) of CD4+CD8+ DP thymocytes (the average number of DP cells in Ox rats was 2.8 time greater than in controls) was found in thymi of peripubertally Ox rats compared to age- and sex-matched control rats, indicating a lasting enhancing effect of ovariectomy on the thymus ability to generate T cells (Figure 1).

*Peripubertal ovariectomy increased thymic T-cell output*

In order to further confirm the ameliorating effect of peripubertal ovariectomy on T-cells generation by thymus of eleven-month-old Ox rats, both CD4+ and CD8+ RTEs were quantified in peripheral blood from these animals. In rats, RTEs can be identified by the unique constellation of surface markers.

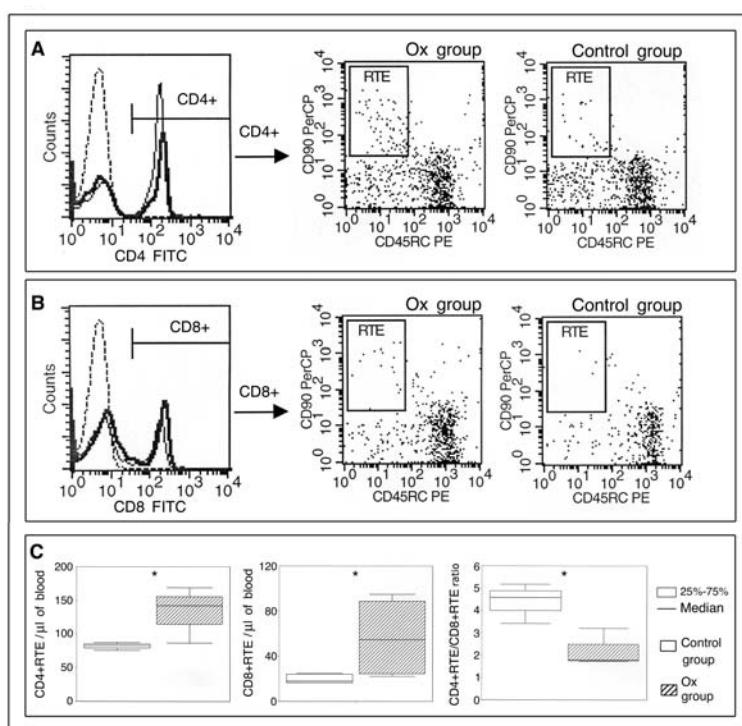


Figure 2. Peripubertal ovariectomy increased the number of CD4+ and CD8+ recent thymic emigrants (RTEs) in eleven-month-old rats. CD4+ and CD8+ cells from (thick line) peripubertally ovariectomized (Ox group) and (thin line) age-matched control rats were gated as it is shown in overlay histograms in Panel A and Panel B, respectively. The gated cells were analyzed for the expression of CD45RC and CD90. The subset of CD45-CD90+ cells (RTEs) is displayed in the upper left region of the dot plot. (Panel C) Box plots represent the number of (left) CD4+ RTEs, (middle) CD8+ RTEs and (right) CD4+ RTE /CD8+ RTE ratio in peripheral blood from eleven-month-old peripubertally ovariectomized (Ox group) and age-matched control rats (Control group). \* $p<0.05$

Namely, these cells have been shown to exhibit CD90+CD45RC- phenotype (Hosseinzadeh and Goldschneider, 1993).

In Ox rats, the numbers of both CD4+ ( $P<0.05$ ) and CD8+ RTEs ( $P<0.05$ ) in peripheral blood were significantly increased compared with corresponding controls. However, since in Ox rats the increase in the number of CD8+ RTEs was more pronounced (the average number was approx. 3-fold greater in Ox rats than in controls) than that in number of CD4+ RTEs (the average number was approx. 1.6 time greater in Ox rats than in controls), a significant drop ( $P<0.05$ ) in CD4+ RTEs/CD8+ RTEs ratio in these animals was registered (Figure 2).

*Peripubertal ovariectomy affected neither the overall number of TCR $\alpha\beta$ + cells nor the numbers of CD4+ and CD8+ T cells*

To get further insight into the impact of the ovariectomy-induced changes on thymic function and peripheral T-cell pool, the size of TCR $\alpha\beta$ + peripheral blood lymphocyte pool (representing the whole number of T cells) and cellularity of CD4+ and CD8+ T-cell compartments were measured (Figure 3). In spite of the increase in the number of both CD4+ and CD8+ RTEs, peripubertal ovariectomy affected neither the overall number of T cells nor the numbers of CD4+ and CD8+ T lymphocytes in peripheral blood from eleven-month-old rats suggesting that, most likely, a homeostatic expansion of memory cells made up for a reduced thymic output in non-Ox control animals (Almeida et al., 2001).

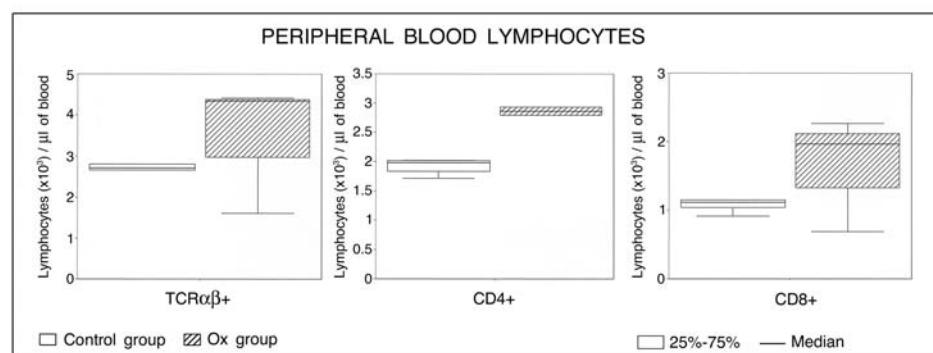


Figure 3. Peripubertal ovariectomy affected neither overall number of TCR $\alpha\beta$ + cells nor the numbers of CD4+ and CD8+ T cells in eleven-month-old rats  
Box plots represent numbers of (left) TCR $\alpha\beta$ +, (middle) CD4+ and (right) CD8+ lymphocytes in peripheral blood from peripubertally ovariectomized (Ox group) and age-matched control rats (Control group)

## DISCUSSION

The present study clearly demonstrated that enhancing effects of gonadal hormone ablation on the thymus weight and cellularity, which have been

registered one month post-peripubertal ovariectomy of AO rats (Leposavić et al., 1996), are maintained until the age of eleven months, so that both thymic weight and thymocyte yield were greater in Ox rats than in controls. In addition, it has been found that the number of CD4+CD8+ DP cells, which is suggested to be an indirect indicator of thymopoietic function (Ferrando-Martinez et al., manuscript in press), and absolute numbers of both CD4+ and CD8+ RTEs were also increased in peripubertally Ox eleven-month-old rats. These findings clearly point to a more efficient generation of T cells by thymi from eleven-month-old rats deprived of ovarian hormones from the age of one month compared with controls.

In favour of our present finding are data that selective estrogen receptor (ER) $\alpha$  agonist, propyl pyrazole triol, in Ox Balb/c mice causes thymic atrophy and changes in thymic CD4/CD8 phenotypic profile lowering the percentage of CD4+CD8+DP thymocytes (Li and McMurray, 2006). Recently, Wang et al. (2008) demonstrated that, in addition to ER $\alpha$ , the membrane ER GPR30 pathway is involved in estrogen-induced thymic atrophy. Multiple mechanisms have been put forward to describe how estrogens cause/accelerate thymic atrophy. First, an accumulating body of evidence indicates that estrogens acting either directly, at multiple developmental steps on developing T cells, or indirectly on thymic epithelial cells to inhibit generation of signalling important for thymocyte survival, may enhance thymocyte apoptosis and thereby induce thymic atrophy (Mor et al., 2001; Hoffman-Goetz et al., 2001; Okasha et al., 2001; Yao and Hou, 2004; Wang et al., 2008). Second, it has been shown that estrogens have an inhibitory effect on thymocyte proliferation *in vitro* (Gulino et al., 1985). Third, it has been strongly suggested that elevated peripheral blood estrogen levels may inhibit T-cell development at multiple stages at both pre-thymic and thymic level, and consequently induce thymic atrophy (Scarpa et al., 1989; 1991; Silverstone et al., 1994; Rijhsinghani et al., 1996; Leposavić et al., 2001; Zoller and Kersh, 2006; Wang et al., 2008). More specifically, decreased level of CD4+CD8+ DP cells significantly contributing to the thymic atrophy after an increase in circulating estrogen levels may be related, not only to increased apoptosis of cells exhibiting this phenotype (Yao and Hou, 2004; Wang et al., 2008), but also to reduction of the number of CD4+CD8+ DP precursors due to adverse effects of elevated circulating estrogen level on T-cell development at multiple points from the bone marrow progenitors to this stage (Scarpa et al., 1989; 1991; Silverstone et al., 1994; Rijhsinghani et al., 1996; Leposavić et al., 2001; Zoller and Kersh, 2006). In other words, it seems quite plausible to suppose that the following mechanisms: i) reduced thymocyte apoptosis, ii) increased thymocyte proliferation, and iii) enhanced T-cell generation are involved in ovariectomy-induced thymic hypercellularity. Moreover, as progesterone is required for complete hormone-(estrogen) and pregnancy-induced thymic involution (Tibbetts et al., 1999), increased thymic weight and cellularity in Ox rats may be related to reduction in progesterone level, as well.

The apparent discrepancy in the duration of effects induced by ovariectomy on the thymus between our study and that performed recently by Min and collaborators (2006) showing in C57BL/6J (B6) mice that the increase in thymic cellularity observed 2 weeks after ovariectomy performed at the age of 4 weeks

completely disappears 20 weeks post-surgery, so that thymic cellularity does not differ between Ox mice and controls, may be explained by species difference. Since the thymic weight and cellularity in peripubertally Ox eleven-month-old rats are markedly less pronounced than in two-month-old rats (Leposavić et al., 1996), i.e. the average thymic weight and cellularity were approx. 2.5 times less in older animals (data not shown), it seems that the initial ovariectomy-induced increase in the thymus weight and cellularity gradually decreases with time. However, to confirm that the effects of peripubertal ovariectomy in rats are longer lasting than in mice, but still transitory, it is necessary to examine the effects of ovarian hormone deprivation in some later time points.

Finally, it should be emphasized, that our study, showing, for the first time to our best knowledge, increased number of both CD4+ and CD8+ RTEs, which is believed to depend solely on thymic T-cell generation, i.e. to be excluded from the peripheral homeostatic control mechanisms (Berzins et al., 1998), in the peripheral blood of eleven-month old Ox rats directly demonstrates that ovariectomy increases thymic T-cell output. Since it has been shown that a reduced number of RTEs in peripheral blood followed by increased number of memory cells is one of the main characteristics of immunological senescence (Ernst et al., 1990; Utsuyama et al., 1992; Kurashima et al., 1995), it seems obvious that peripubertal ovariectomy markedly postpones chronobiological ageing of the immune system in rats. However, it should be pointed that this increase in number of RTEs was not followed by a proportional rise in the overall number of T cells. This finding may be explained by reduced proliferation of memory cells in Ox rats. Namely, it has been shown that aged animals faced with a decreased thymic output, on one side, and homeostatic pressure to fill vacant spaces ("niches"), on the other side, respond by increased proliferation of memory CD4+ and CD8+ T cells providing maintenance of a relatively steady number of peripheral blood T cells (Freitas and Rocha, 1993; 2000), but leading to an accumulation of replicative-senescent T cells (as T cells have a finite replicative lifespan) and a significant narrowing of peripheral TCR repertoire (Mosley et al., 1998; LeMaoult et al., 2000; Miller et al., 2000). Therefore, although the overall number of T cells in peripheral blood was not significantly affected by ovariectomy, qualitative alterations in the T-cell pool enabling a more efficient response to newly encountered antigens may be expected in Ox rats. The significantly increased number of CD8+ than CD4+ RTEs is fully in agreement with well-known enhancing effect of estrogens on CD4+ – mediated immune response (Grossman, 1985).

#### **CONCLUSIONS**

In conclusion, taken as whole, our results suggest that, even if the peripubertal rise in circulating levels of ovarian hormones is not causal or not solely the causal factor of thymic involution, the maintenance of physiological levels of these hormones in the circulation is necessary, at least, to provide regular dynamics of the thymic involution process, which is evoked by some other factors that still remain to be identified. Furthermore, the study indicates, that withdrawal

of ovarian hormone action from the peripubertal stage of development in rats may, at least, for a long period of time, prevent/mitigate age-associated decline in T-cell – mediated immune response.

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**PERIPUBERTETNA OVARIJEKTOMIJA OBEZBEĐUJE DUGOTRAJNO ODLAGANJE  
STARENJEM USLOVLJENOG SMANJENJA CELULARNOSTI TIMUSA I  
PRODUKCIJE T LIMFOCITA**

PERIŠIĆ MILICA, KOSEC D, PILIPOVIĆ I, RADOJEVIĆ KATARINA, PEŠIĆ VESNA,  
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**SADRŽAJ**

Ova istraživanja su preduzeta sa ciljem da se preispita uloga gonadnih hormona u involuciji timusa, koja je nedavno dovedena u pitanje. U tom cilju je kod 11 meseci starih ženki pacova, koje su ovarijskotomisane (Ox) u peripubertetnom uzrastu, analizirana: i) težina i celularnost timusa, ii) broj CD4+CD8+ dvostruko pozitivnih (DP) timocita, za koji se smatra da odražavaju sposobnost organa da generiše zrele T limfocite, iii) broj neposrednih emigranata iz timusa (RTE) i iv) ukupan broj CD4+ i CD8+ limfocita u perifernoj krvi. Dokazano je da su težina i celularnost timusa bile značajno veće u Ox životinja. Kod ovih životinja je nađen i povećan broj DP timocita, kao i CD4+ i CD8+ RTE, što ukazuje na efikasniju produkciju T ćelija u njihovom timusu. Ovaj nalaz, u kontekstu nepromjenjenog broja CD4+ i CD8+ ćelija u perifernoj krvi, takođe sugerije smanjenu homeostatsku proliferaciju memorijskih ćelija, odnosno ukazuje na kvalitativne promene u perifernom T ćelijskom repertoaru (koje obezbeđuju efikasniji odgovor na nove antogene) bez kvantitativnih promena. U celini, rezultati ukazuju da u odsustvu hormona ovarijuma počevši od ranog peripubertetnog uzrasta dolazi do značajnog odlaganja/ublažavanja involucije timusa i posledičnih promena na periferiji.