Histol Histopathol (2011) 26: 157-166

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Somatostatin 14 affects the pituitary-ovarian axis in infant rats

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Summary. The effects of multiple somatostatin (SRIH-14) treatment on the pituitary-ovarian axis were examined in infant rats. Female Wistar rats received subcutaneously two daily 20 μg/100g b.w. doses for five consecutive days (from 11 to 15 days of age). Changes in cell volume, volume density and number per unit area (mm^2) of follicle-stimulating (FSH), luteinizing (LH) and somatotropic (GH) immunolabeled cells were evaluated by stereology and morphometry. Serum FSH and LH concentrations were determined by RIA. Ovaries were analyzed by simple point counting of follicles. SRIH-14 treatment significantly reduced FSH and LH cell volume, while their volume density and number *per* unit area were unaltered. Serum concentrations of FSH and LH were significantly reduced. Volume and volume density of GH cells was significantly decresed after SRIH-14 treatment, while their number *per* unit area was unaltered. In the ovary, SRIH-14 induced a significant increase in the percentage of primordial follicles followed by a significant decrease in percentage of primary follicles. The number of healthy and atretic preantral follicles was unchanged. It can be concluded that SRIH-14 treatment during the infantile period markedly inhibits pituitary FSH, LH and GH cells. In the ovary, SRIH-14 acts by inhibiting initial folliculogenesis without affecting atretic processes.

Key words: Gonadotropic cells, Somatostropic cells, Ovary, Folliculogenesis, Stereology

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Introduction

Somatostatin (somatotropin release inhibiting hormone, SRIH) is a tetradecapeptide hormone that is widely distributed throughout the central nervous system (CNS) and peripheral tissues in mammals (Reisine and Bell, 1995). SRIH potently inhibits basal and stimulated secretion from a wide variety of mammalian endocrine and exocrine cells associated with growth, development and metabolism. In the CNS it functions as a neurotransmitter/neuromodulator. It also possesses an anti-proliferative action (Patel, 1999). In the pituitary, besides inhibiting growth hormone (GH) secretion, it blocks the release of other adenohypophyseal hormones, including thyrotropin (TSH; Sekulić et al., 2000), prolactin and, in some conditions, adrenocorticotropin (ACTH; Lamberts, 1988; Starčević et al., 2000).

In the hypothalamo-pituitary-gonadal axis SRIH reduces serum concentration of luteinizing hormone (LH), but has little or no effect on the release of follicle-stimulating hormone (FSH) in women with polycystic ovary syndrome (Prelević et al., 1990), or in male rats (Starčević et al., 2002; Milošević et al., 2004). In female rats, it inhibits both types of gonadotropic cells (Nestorović et al., 2001, 2004, 2008). *In vitro* studies have shown that SRIH affects steroidogenesis in ovarian granulosa and granulosa lutein cells (Rajkumar et al., 1992; Andreani et al., 1995; Holst et al., 1995, 1997). Expression of the pre-prosomatostatin gene was demonstrated in the ovary of the orange-spotted grouper, and this was increased by estradiol (Zhang et al., 2009). However, the RT-PCR performed to detect mRNAs for SRIH in the mouse ovary gave inconstant and inconsistent results, suggesting that the SRIH mRNAs were either absent or present very weakly (Gougeon et

al., 2010).

Additionally, somatostatin receptors (sstr) were found in human granulosa (Strauss et al., 2003; Adams et al., 2004) and granulosa lutein cells (Strauss et al., 2003), and in mouse granulosa cells and oocytes (Gougeon et al., 2010).

The functionality of the hypothalamo-pituitary-growth hormone and hypothalamo-pituitary-gonadal axes are closely related, and age dependant (Hull and Harvey, 2001, 2002). The infantile period of life in female rats (8-21 days of age) is characterized by high blood concentrations of FSH. It rises from 5 days after birth, reaching maximum concentrations on day 12, followed by a marked decline through postnatal day 25 (Dohler and Wuttke, 1975; Taya and Sasamoto, 1988). LH increases shortly after birth to a maximum on day 12 and then declines through the end of the juvenile period (Ojeda and Ramirez, 1972; Dohler and Wuttke, 1975). High concentrations of gonadotropic hormones in the blood are partly the result of very marked FSH and LH responsiveness to the positive influence of luteinizing hormone-releasing hormone (LHRH; Ojeda et al., 1977; Taya and Sasamoto, 1988), and low responsiveness to the negative influence of endogenous estrogen (Meijs-Roelofs and Kramer, 1979). Between days 5 and 12, concentration of GH in developing female rats is significantly higher than in adult animals (Ojeda and Jameson, 1977). During the second week after birth rat ovaries are under strong gonadotropin influence (Lunenfeld et al., 1975; Hage et al., 1978). Almost twice as many follicles begin to move into more advanced developmental stages during the second postnatal week than at later ages (Ojeda and Urbanski, 1994).

We have previously shown that SRIH-14 inhibits both types of gonadotropic cells in the pituitary and has a negative effect on the process of folliculogenesis in the ovaries of adult (Nestorović et al., 2001, 2004) and peripubertal rats (Nestorović et al., 2008). However, the influence of SRIH-14 on the pituitary-ovarian axis during the infantile period of life has been less well examined. The aim of this study was to examine the effects of SRIH-14 on the morphological and functional features of pituitary gonadotropic cells, the immunocytochemical and morphometric characteristics of somatotropic cells and on follicular development in the ovaries of infant rats.

Materials and methods

Time-mated pregnant Wistar rats were housed individually and maintained in a controlled environment (12 h light:12 h dark; 22±2°C), with food (Veterinarski Zavod 'Subotica', Subotica, Serbia) and water *ad libitum*. Female pups were injected s.c. twice daily (8 AM and 8 PM) with 20 μg SRIH-14 (S9129, Sigma, St. Louis, Mo., USA) per 100 g body weight (b.w.) for five consecutive days between 11 and 15 days of age. The dose regimen was based on that of Rebuffat et al., (1984) except that SRIH-14 was administered every 12 h instead of every 8 h (Nestorović et al., 2001). Control female pups s.c. received the equivalent volume of saline by the same schedule. Animals were sacrificed at 8 AM on the 16th day of life. Blood was collected from individual animals and the sera were stored at -70°C until FSH and LH determination. The experimental protocols were approved by the local Animal Care Committee. They conformed to the recommendations given in the "Guide for the Care and Use of Laboratory Animals" (National Academy Press, Washington D.C. 1996).

After removal, the pituitaries and ovaries were weighed. The pituitaries were fixed in Bouin's solution, dehydrated and embedded in paraffin wax. Pituitary sections (5 μm thick) were immunocytochemically stained. The ovaries were immersed either in Bouin's solution or in 4% glutaraldehyde in phosphate buffer (pH 7.4). The samples fixed in Bouin's fixative were embedded in paraffin, serially sectioned at a thickness of 6 μm and stained with hematoxylin and eosin (H&E). The samples immersed in 4% glutaraldehyde were cut into small blocks, left in the fixative for 24 h at 4°C, postfixed in 1% osmium tetroxide and then embedded in araldite. Sections (1 μm thick) were cut, stained with 1% methylene blue and viewed by light microscopy.

Pituitary

Immunocytochemistry

Series of seven sections cut through three tissue levels (dorsal, middle and ventral portions) of the *pars distalis* were used for immunostaining. After rehydration, the sections were stained immunocytochemically. Gonadotropic cells were visualized using the peroxidase–antiperoxidase (PAP) method, as previously described (Nestorović et al., 2001). Anti-rat ßFSH (1:300 v/v) or anti-rat ßLH polyclonal antibodies (1:500 v/v) (National Institute of Health, Bethesda, MD, USA) served as the primary antibodies. For detection of somatotropic cells, sections were incubated with polyclonal anti-human GH antibodies (1:300 v/v (DAKO A/S, Glostrup, Denmark)) (Nestorović et al., 2008).

Stereological measurements

Immunocytochemically stained 5 μm thick sections of the *pars distalis* were used for morphometric examinations of FSH, LH and GH cells that possessed visible nuclei. Cell volumes (Vc) and volume densities (V_V) were estimated by light microscopy at 1000x magnification using the M_{42} multipurpose test system (Weibel, 1979). Cell volumes were expressed in μm^3, while volume densities were given as percentages of total pituitary cells per mm^3. At the same time, the number of immunoreactive cells per unit area (mm^2) in each section was analyzed.

Ovary

Morphometry and classification of the ovarian follicles

Every second section of the ovaries that were embedded in paraffin (6 μm thick) was examined by light microscopy. The number of follicles was determined by simple point counting. In the ovaries of 16-day-old females only small, preantral, follicles were present. The follicles were divided into six classes as defined by Gaytan et al. (1998) as follows: (1) primordial follicles containing an oocyte surrounded by a layer of 3-6 flattened pre-granulosa cells; (2) primary follicles consisting of an oocyte surrounded by a layer containing one enlarged cell or a whole layer of cuboidal pre-granulosa cells; (3) multilaminar class A follicles (Ma) with 1-2 layers of granulosa cells measuring up to 75 μm in diameter; (4) multilaminar class B follicles (Mb) measuring from 76 to 150 μm; (5) multilaminar class C follicles (Mc) measuring from 151 to 200 μm; and (6) multilaminar class D follicles (Md) measuring from 201 to 274 μm. Follicle diameters were measured (two diameters at right angles) in the largest cross section containing the oocyte nucleolus. In the ovaries of 16-day-old rats Md follicles were absent, and Mc follicles were rare. Atretic follicles exhibited alterations in the granulosa layer and/or on the oocyte. Follicles that contained pycnotic granulosa cells in abundance and/or degenerated oocyte were classified as atretic. Oocyte degeneration was reflected in highly irregular shape and/or the presence of pycnotic nucleus.

Determination of serum hormone concentrations

Serum concentrations of FSH and LH were measured with commercial radioimmunoassay (RIA) kits (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK). Intra-assay and inter-assay coeffcients of variation were 4.2 and 7.9% for FSH and 6.5 and 10.9% for LH, respectively.

Statistical analysis

All results were expressed as means for five animals per group ± standard deviation (SD). The data were tested for normality of distribution by the Kolmogorov-Smirnov test, whereas the homogeneity of variances was evaluated by Leven's test. Student's t-test was used to compare the mean values. The minimum level of statistical significance was set at $P<0.05$. For each follicle type, the percentages of healthy primordial, primary, Ma, Mb and Mc follicles were calculated and compared between control and treated group.

Results

Body and organ weight

Somatostatin 14 applied in the infantile period of life did not lead to differences in body weight, pituitary or ovarian weights compared to the control values (Table 1).

Table 1. Body, pituitary and ovary weights of control and SRIH-14 treated females.

	Body weight (g)
Control	26.1±2.4
SRIH-14	25.3±1.9
	Pituitary weight (mg)
Control	2.4±0.5
SRIH-14	2.5±0.5
	Ovary weight (mg)
Control	3.9±0.7
SRIH-14	4.1±0.9

All values are given as mean ± SD (n=5).

Pituitary

Follicle-stimulating (FSH) and luteinizing (LH) cells in the pituitary of control infant females were numerous, positioned throughout the *pars distalis*, present in groups or occasionally alone, often in close contact with blood capillaries. They were strongly immunocytochemically stained, large, polygonal, oval or polyhedral in shape with prominent, often eccentrically located nuclei (Fig. 1a,c). After SRIH-14 treatment, gonadotropic cells were smaller in size (Fig. 1b,d). In the pituitaries of both control and treated females, FSH and LH cells were stained homogenously and the intensity of immunostaining varied from weak to very intense. Stereological anlaysis showed that SRIH-14 treatment reduced FSH and LH cell volume in comparison to control values, by 12.6% ($P<0.05$) and 19.6% ($P<0.05$), respectively (Fig. 2a). The volume density of gonadotropic cells (Fig. 2b), and their number per unit area (mm^2; Fig. 2c) were not changed.

Somatotopic (GH) cells were abundant in the pituitaries of control females, positioned throughout the *pars distalis*, mostly in groups, with strongly and homogenously immunocytochemically stained cytoplasm. Their shape ranged from ovoid to pyramidal (Fig. 1e). After SRIH-14 treatment, GH cells were smaller in size, with cytoplasmic immunostaining that varied from weak to very intense (Fig. 1f). The volume of GH cells was significantly reduced by 27.7% after SRIH-14 treatment (Fig. 2a). Compared to the controls, SRIH-14 injections led to a decrease of GH cell volume

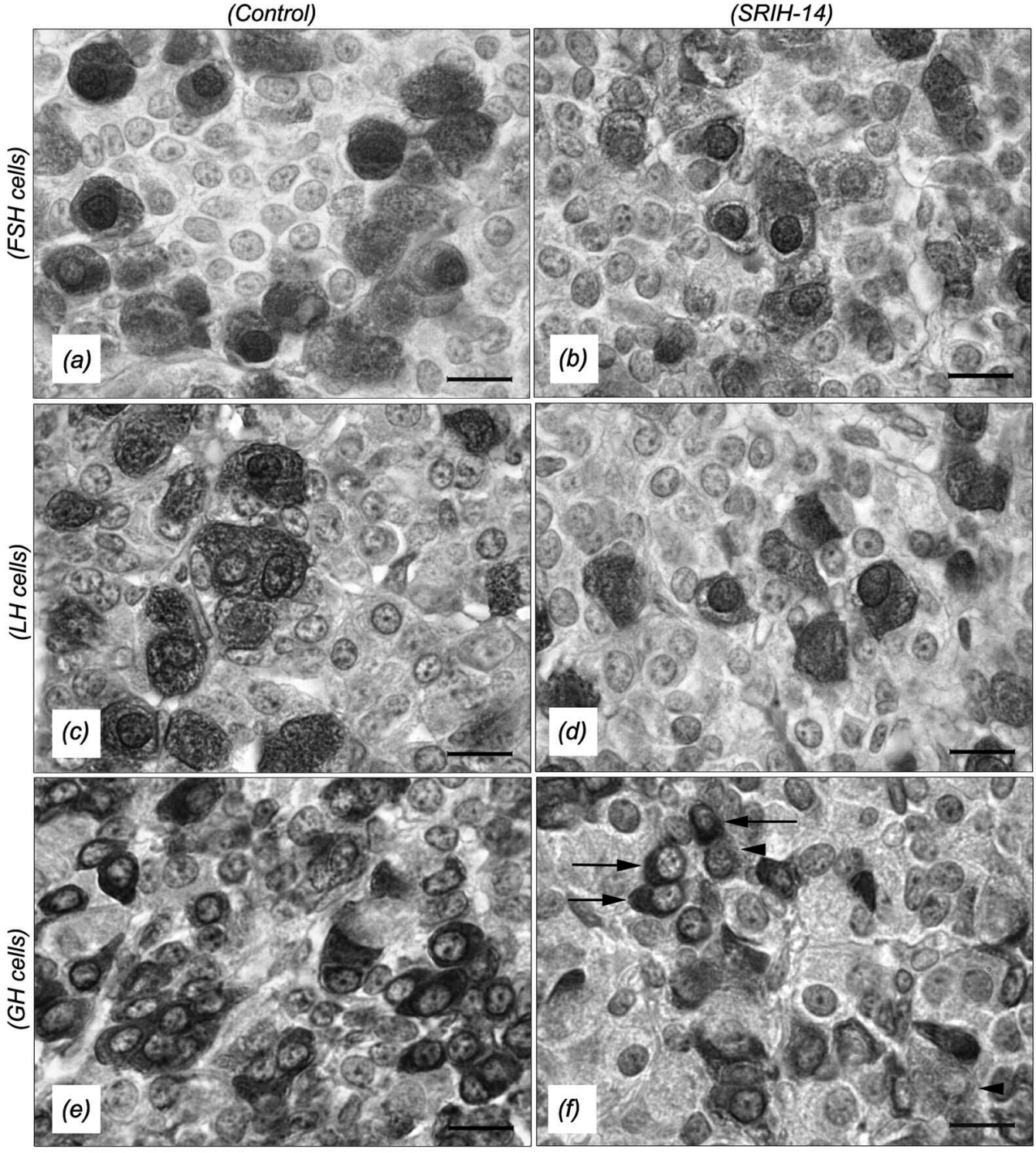

Fig. 1. Representative micrographs of gonadotrops (**a, b, c, d**) and somatotrops (**e, f**) from control (**a, c, e**) and SRIH-14-treated infant female rats (**b, d, f**). In the pituitaries of SRIH-14-treated rats, FSH (**b**) and LH (**d**) cells were smaller and different in shape. GH cells (**f**) were smaller with cytoplasmatic immunostaining that varied from weak (arrowheads) to very intense (arrows). Bar: 10 μm.

density (by 28.3%; P<0.05; Fig. 2b). The GH cell number per unit area was not significantly changed (9.9%; P>0.05; Fig. 2c).

Serum FSH and LH concentrations

After the SRIH-14 treatment, mean serum FSH concentration was lower by 19.1% (2.88±0.31 vs. 3.55±0.31; P<0.05). SRIH-14 lowered serum LH concentration as well, by 36.9% (1.47±0.48 vs. 2.33±0.05; P<0.05).

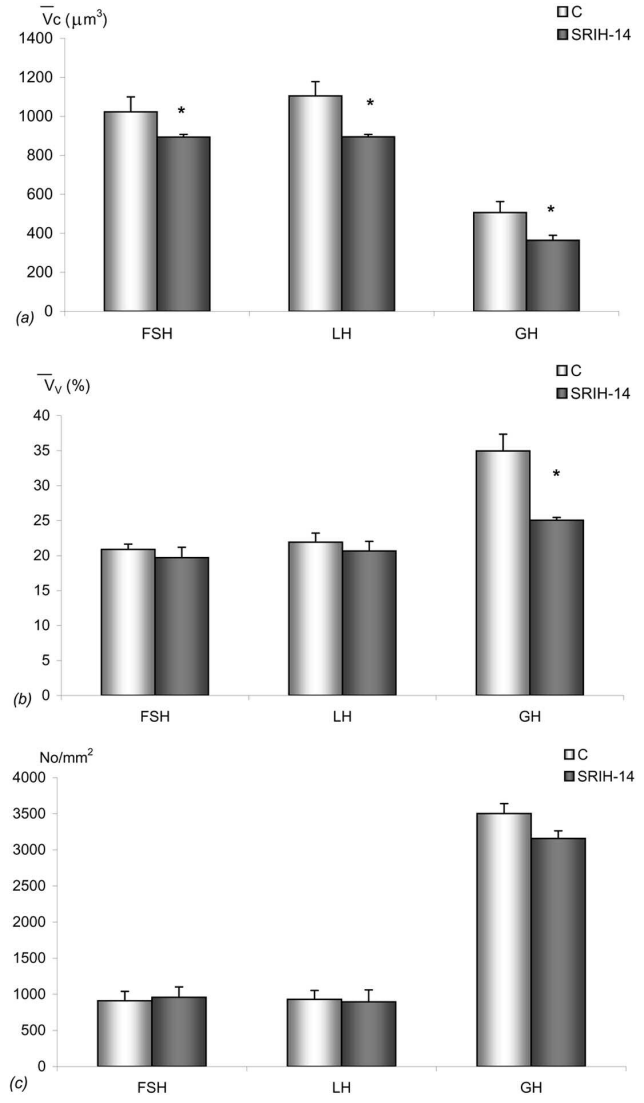

Fig. 2. a. The cellular (Vc) volume (μm^3). **b.** Volume density (V_v; %). **c.** Number (No) per unit area (mm^2) of FSH-, LH- and GH-immunopositive cells in female control (C) and SRIH-14-treated rat pups. All values are given as mean ± SD (n=5); *P<0.05.

Ovary

Numerous growing follicles were present in control rat ovaries on day 16. Most of them corresponded to Mb follicles (Fig. 4a,b), one of which is shown in Fig. 4c. Low numbers of Mc follicles were found in control infant rat ovaries. The structure of ovaries from SRIH-14 treated females was also well organized with all developmental stages present (Fig. 4d). However, an abundance of primordial, quiescent follicles was observed (Fig. 4e). Nevertheless, semi-thin sections revealed normal follicle morphology in the ovaries of SRIH-14 treated animals (Fig. 4f).

Morphometric analysis showed that the number of primordial follicles was significantly increased (by 50.1%; P<0.05; Table 2) as well as their percentage in the ovaries of SRIH-14 treated females, compared to the controls (Fig. 5). The number of primary follicles was lower, but due to large inter-individual differences, not significantly (by 11%; P>0.05; Table 2). However, when expressed as percentage of all ovarian follicles, a significant decrease (P<0.05) was noted in the ovaries of

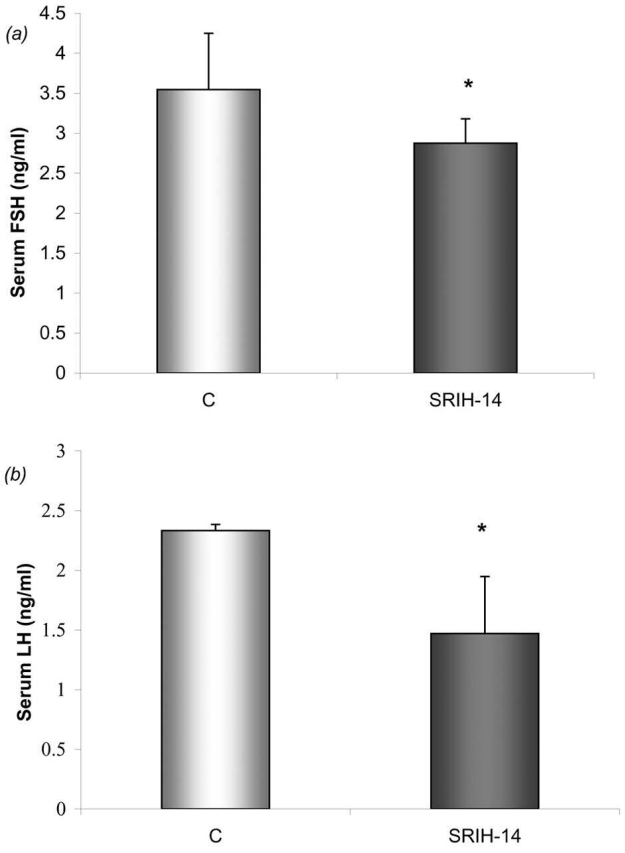

Fig. 3. Serum concentrations of FSH **(a)** and LH **(b)** in control (C) and SRIH-14-treated infant female rats. All values are given as means ± SD (n=5); *P<0.05.

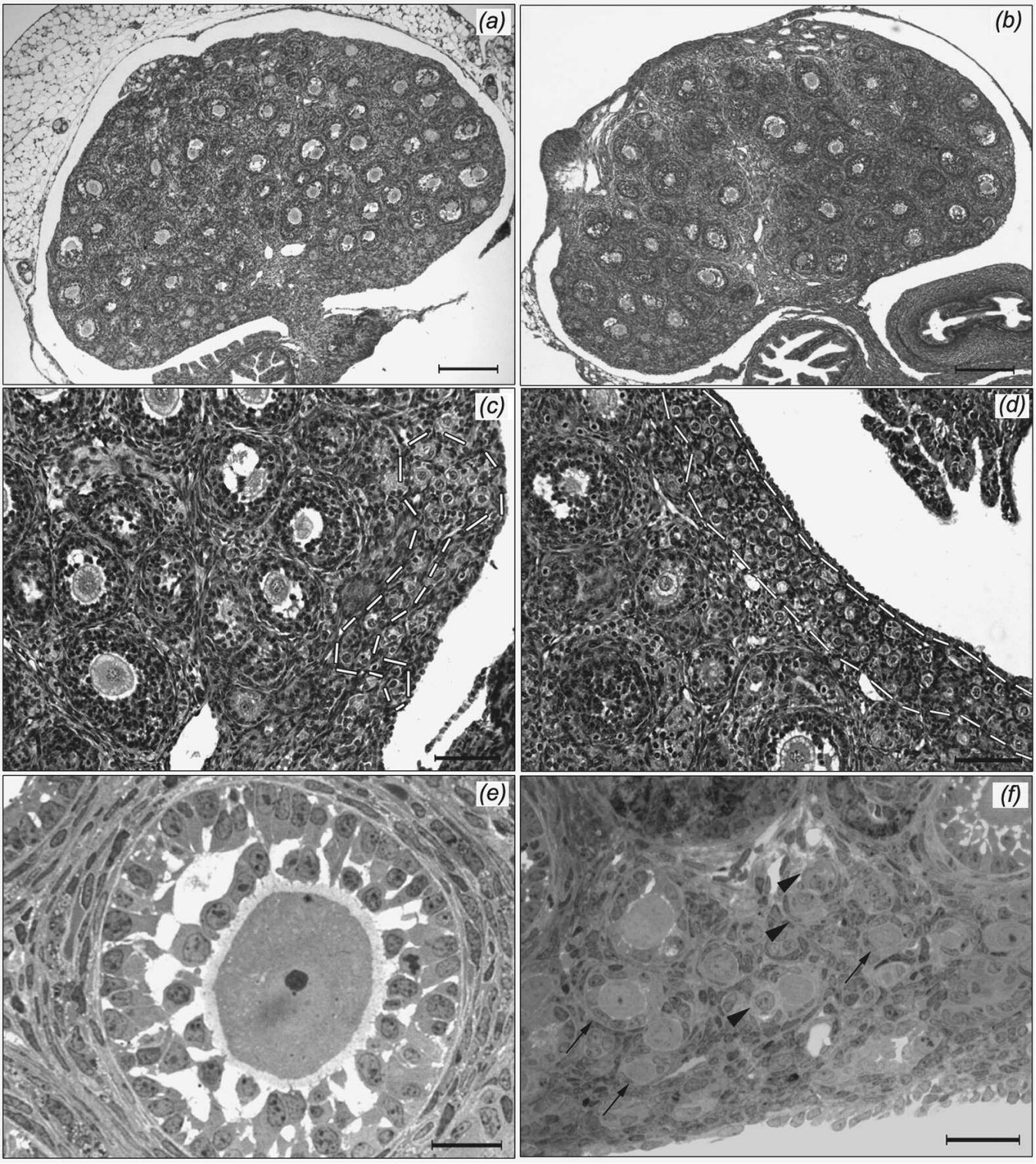

Fig. 4. Representative micrographs of ovaries of control **(a)** and SRIH-14-treated rat females **(b)**. Numerous healthy growing follicles (Ma and Mb) in the ovary of a control **(c)** 16-day-old female. In the ovary of a SRIH-14 treated female **(d)** the primordial follicles are more abundant (dashed line). A healthy growing follicle in the ovary of a control female **(e)**. Primordial (arrowheads) and primary (arrows) follicles in the ovary of a SRIH-14 treated female **(f)**. a-d -H&E; e, f - methylene blue. Scale bars: a, b, 200 μm; c, d, 70 μm; e, 20 μm; f, 30 μm.

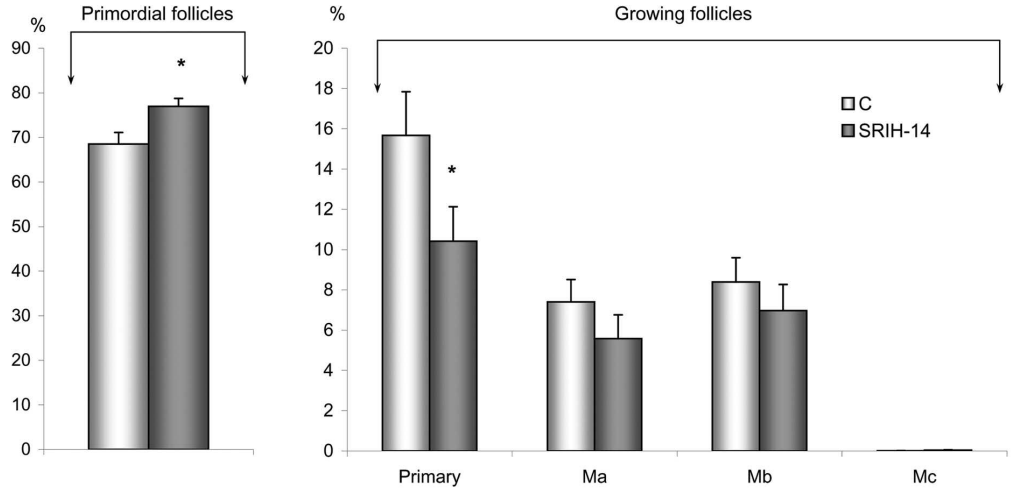

Fig. 5. The percentage of healthy primordial and growing follicles per ovary of control (C) and SRIH-14-treated infant rats. All values are given as mean ± SD (n=5); *P<0.05.

Table 2. The average number of healthy follicles in the ovaries of control and SRIH-14 treated females.

Follicle class	Control	SRIH-14
Primordial	1176±190	1765±350*
Primary	269±26	239±54
Ma	127±28	128±24
Mb	144±27	160±27
Mc	0±0.4	1±0.6

All values are given as mean ± SD (n=5); *P<0.05.

Table 3. The summarized number of healthy and atretic small growing follicles and the healthy to atretic follicle number ratio, in the ovaries of control and SRIH-14 treated rats.

	C		SRIH-14	
	No. of follicles	Ratio	No. of follicles	Ratio
Healthy small growing follicles (Σ)	540±73	10:1	528±68	9:1
Atretic small follicles (Σ)	56±8		60±9	

All values are given as mean ± SD (n=5).

SRIH-14 treated females when compared to the control values (Fig. 5). No change was observed in the number of Ma and Mb follicles (Table 2), or their percentages (Fig. 5). In summary, the number of healthy growing follicles and the number of atretic follicles in the ovaries of SRIH-14 treated females was not different from values for the controls. The healthy to atretic follicle ratio was similar in both groups (Table 3).

Discussion

The results of this study demonstrate that somatostatin, administered to infant female rats, acted in an inhibitory manner on pituitary gonadotropic and somatotropic cells. It also affected the process of folliculogenesis in the ovary.

The suppressive effect of SRIH on pituitary hormone secretion is age dependant. Immature pituitaries are relatively resistant to the action of SRIH and increasing sensitivity to SRIH with advancing age is believed to cause characteristic developmental changes in pituitary hormone secretion in mammals (Rieutort, 1981; Khorram and McCann, 1984; Cuttler et al., 1986; Silverman et al., 1989). The relative resistance to SRIH of female infant rat pituitaries was reflected in their unchanged weight. When given to peripubertal or adult females, SRIH-14 significantly reduced absolute pituitary weight (Nestorović et al., 2008, 2001). As observed earlier for neonatally treated females (Nestorović et al., 2006), the body weight of our treated infant females did not differ from controls.

The biological effects of SRIH are mediated through five distinct receptor subtypes ($sstr_{1-5}$). All five receptor mRNAs are expressed in the rat pituitary and this is developmentally determined in a subtype-specific manner (Reed et al., 1999). The expression of $sstr_2$ mRNA and protein rises markedly after birth and reaches a maximum in the adult. In the pituitaries of 12-day-old rats, the expression of $sstr_2$ mRNA was significantly lower than in 30 or 45-day old rats. The abundance of $sstr_1$, $sstr_3$, $sstr_4$, and $sstr_5$ mRNAs in the rat pituitary does not change with age (Reed et al., 1999). SRIH-14, used in this study, has the same affinity for all types of sstrs (Patel, 1999).

In our experimental conditions SRIH-14, administered daily from 11 to 15 days of age, had an

inhibitory effect on both types of gonadototropic cells, which was reflected in the reduction of their cell volumes and decreased concentrations of circulating FSH and LH. It must be noted, however, that multiple SRIH-14 treatment at this stage of life inhibited gonadotropic cells to a lesser extent than in peripubertal (Nestorović et al., 2008) or adult rats (Nestorović et al., 2001). After SRIH-14 treatment in the infantile period, cytoplasms of FSH and LH cells were homogenously stained and their volume densities were not altered, in contrast to the morphologically changed gonadotrops after SRIH-14 application to older rats. This effect of SRIH-14 could be the result of a direct effect on the pituitary through sstrs, and indirect action through inhibition of gonadotropin-releasing hormone (GnRH). The somatostatin analog, octreotide, was shown to inhibit activation of GnRH-containing cells in the hypothalamus of ovariectomized rats (Van Vugt et al., 2004) and expression of $sstr_{2-4}$ mRNA was detected in mouse GnRH neurons (Todman et al., 2005).

Blood concentration of GH is very high between day 5 and 12 in the developing female rat, while the pituitary GH concentration is low (Ojeda and Jameson, 1977). In this study, volume and volume density of GH cells were were significantly decreased after multiple SRIH-14 treatment. These results are in acorrdance with our previous study where neonatally applied SRIH-14 markedly decreased number, volume and volume density of GH cells. These changes were sustained until adulthood (Nestorović et al., 2006).

In the ovaries of SRIH-14 treated females, the number and percentage of primordial, quiescent, follicles was significantly increased, which was accompanied by a lower percentage of primary follicles. The number of preantral and/or healthy atretic follicles of all examined classes was unchanged, as well as the healthy/atretic follicle ratio, when compared to the controls. The unchanged number of larger follicles resulted in similar ovarian weights. This indicates that SRIH-14 in infant rats inhibited the critical step in folliculogenesis, i.e. transition from a non-growing to a growing pool of follicles, only. Enlargement of the non-growing pool of follicles was observed when SRIH-14 was applied chronically to immature, or multiply to peripubertal females (Nestorović et al., 2004, 2008), but not if it was applied multiply in adult period (Nestorović et al., 2001). This suggests that the period of life when SRIH-14 is administered determines the effect of this hormone on non-growing pool of follicles.

The mechanisms that regulate initial process of folliculogenesis remain unclear. Resting follicles are likely to be under constant inhibitory influence of systemic and/or local origin to remain dormant, since removal of ovarian tissue and its fragmentation causes the primordial follicles to move en mass toward a stage of late primary follices (Wandji et al., 1997). There is evidence that signals from the pituitary are important since decreased initial recruitment was observed in hypophysectomized rodents (Wang and Greenwald, 1993), and that FSH, together with some neurotransmitters, in particular vasoactive intestinal polypeptide (VIP), stimulates early folliculogenesis (Mayerhofer et al., 1997). There is evidence that even disturbance of the somatotropic axis can result in reduced initial recruitment of follicles (Slot et al., 2006). An opposite effect, i.e. stimulation of initial recruitment of follicles, was observed after *in vitro* treatment with SRIH antagonist (Gougeon et al., 2010). Gougeon and coworkers (2010) indicate that enlargement of a non-growing pool of follicles in the ovaries of SRIH-14 treated females, could be the consequence of a direct effect of somatostatin on the ovary. These authors documented the presence of $sstr_2$ and $sstr_5$ on granulosa cells in the infant mouse ovary.

To conclude, application of SRIH-14 to infant female rats exhibited a significant inhibitory effect on pituitary gonadotropic and somatotropic cells. In the ovaries it inhibited initial folliculogenesis without affecting more advanced developmental stages or stimulating atretic processes. These results indicate that SRIH might be used to prevent premature exhaustion of the ovarian reserve in young females showing a familial antecedent of premature ovarian failure.

Acknowledgements. This work was supported by the Ministry for Science of Serbia, Grant number 143007B. The authors wish to express their gratitude to Dr. Anna Nikolić, Ph.D. for language correction of the manuscript and Ivan Vasiljević, Olympus, Belgrade, Serbia, for technical support.

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Accepted July 20, 2010