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Full Research Article

OVARIAN SURFACE EPITHELIUM OF HYPOTHYROID NEWBORN AND NEONATAL RATS: FROM PROLIFERATING CELL NUCLEAR ANTIGEN AND CASPASE-3 PERSPECTIVES

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Abstract

Introduction. The ovarian surface epithelium (OSE) undergoes intensive regeneration and remodeling after each ovulation during the whole reproductive period. This process increases the risk of one of the most common ovarian tumors in women and the female dog. Considering the fact that maternal hypothyroidism highly impacts cell proliferation and cell death during folliculogenesis in the early neonatal period, we aimed to analyze its effect on OSE morphology and dynamics.

Materials and Methods. The study was performed on newborn (24-h-old) and neonatal (4-day-old) female rats, a randomized trial between the control and hypothyroid groups, born under controlled circumstances and hypothyroid mothers, respectively. Their ovaries were analyzed histologically and processed to determine the OSE cell height as an average value of four measurement points. Also, the immunopositivity of the proliferating cell nuclear antigen (PCNA) and caspase-3 were assessed semiquantitatively.

Results and Conclusions. No major structural differences of OSE were found between groups within the given ages except for a slight increment of OSE cell height and incompleteness of apical cell membrane with cytoplasmic projections in hypothyroid animals. PCNA immunopositivity of the OSE cells was higher in ovaries of hypothyroid animals of both ages in comparison to the controls. Moreover, only scarce OSE cells were caspase-3 positive in both groups and ages, with no difference in immunopositivity. Our study confirms the impact of hypothyroidism in the early postnatal period on

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morphology and proliferation rate of OSE cells, with no effect on caspase-3 dependent cell removal, which may serve as a premise for future investigation of potential carcinogenesis, in terms of prevention and treatment of ovarian cancer.

Key Words: Apoptosis, maternal hypothyroidism, proliferation

INTRODUCTION

The epithelium that covers the ovaries (ovarian surface epithelium; OSE) is not a simple peritoneal mesothelium, but rather a very dynamic structure composed of somatic cells which enable regeneration and remodeling to occur following each ovulation. Also, the OSE is recognized as a possible source of germinative stem cells (Yazdekhasti et al., 2016). Moreover, OSE is a cancer-prone stem cell niche (Kang et al., 2013) and a source of most ovarian tumors in women and female dogs (Auersperg et al., 2008; Fong & Kakar, 2010; Banco, 2011).

The decrease of thyroid hormones during the prenatal period leads to atresia of ovaries in pubertal rats (Radovanović et al., 2012). In early postnatal hypothyroid pups, oocytes have an increased PCNA expression with no visible markers of apoptosis (Danilović Luković et al., 2016; 2017). PCNA reflects the proliferation of the cells (Xu et al., 2011) and as such is recorded in the OSE cells of the mouse ovary during the first postnatal week (Li, 1994). PCNA is also involved in DNA replication and reparations (Kurki et al., 1988) and it may indicate a susceptibility of the cells to cancer development (Anreder et al., 1999; Giles et al., 2006). On the other hand, during ovulation, the cells of OSE in the region of the stigma become apoptotic (Murdoch, 2000). Apoptosis is significant in the germ cells' elimination during fetal development (De Felici et al., 2008), follicle atresia and removal of the granulosa cells in adult animals (Tilly & Robles, 1999). In the early postnatal period, in addition to apoptosis, other forms of cell death in the ovaries have been defined (Escobar et al., 2008; Danilović Luković et al., 2016; 2017).

Scarce data are available on the histology of OSE in the early neonatal period (Rajah et al., 1992). Having in mind that the OSE cells express nuclear receptors for thyroid hormones (TH) (Aghajanova et al., 2009), it would be interesting to investigate if decrease of these hormones modifies OSE proliferation and survival.

The aim of this study was to determine changes in OSE histomorphology and expression of proliferation (PCNA) and apoptosis (caspase-3) markers in the ovaries of newborn (in the first 24 hours after birth) and neonatal (four days after birth) rats due to maternal hypothyroidism. This period in the development of the ovaries is characterized by intense proliferative activity and the formation of the tunica albuginea that separates this epithelium from the cortex (Rajah et al., 1992). Our investigation will broaden the already existing knowledge about the OSE histology and the dynamics in light of neonatal hypothyroidism and may be a step forward in the understanding of the eventual link between hypothyroidism and the risk of ovarian cancer.

MATERIALS AND METHODS

Animals and experimental protocol

Female Albino Oxford rats aged 3 months were housed in the animal facility under standard laboratory conditions with a cycle of 12 h light: 12h darkness and food and water intake *ad libitum*. After mating, the presence of sperm in the vaginal smears was considered as gestational day 0.

Dams were randomized into two groups, each consisting of six animals. Treated mothers were given 1.5 mg/L 6-n-propyl-2-thyouracil (PTU) (Sigma Chemical Co. St. Louis, MO, USA) in drinking water from the beginning of pregnancy and during lactation. Controls consumed tap water without PTU. Five newborn (within first 24 hours after birth) and five neonatal (four-day-old) female pups from treated mothers and another ten matched by age from control mothers were euthanized. Hypothyroid status of mothers and pups was established in our previous research (Danilović Luković et al., 2016; 2017).

The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade, according to the guidelines issued by the EU registered Serbian Laboratory Animal Science Association implementing the European Communities Council Directive (2010/63/EEC) and the rules for good laboratory practice established by the EU and the OECD. All experimental procedures were performed under the supervision of a licensed veterinarian, who specialized in the conduct of experiments on laboratory animals. All researchers who were part of the study were authorized to perform experiments in laboratory animals.

Tissue processing and sampling for light microscopy

The ovaries were removed and fixed in 10% neutral-buffered formalin for 24 h at room temperature, dehydrated, and embedded in paraffin. Histological analyses were performed on 5μm thick serial sections stained with hematoxylin/eosin. Every fifth section of the ovary was analyzed.

OSE height measurement

The height of the epithelium was measured at four points, corresponding to the position of the small hand on the clock when it is set at 12h, 3h, 6h, 9h, as previously described (Talsness et al., 2015). OSE height was determined as the average value of the four measurements for each ovary.

Immunohistochemistry protocols and examination

Immunohistochemistry was performed on paraffin sections by using anti-PCNA (sc-7907) (Santa Cruz, Biotech, USA) and anti-cleaved caspase-3 (Asp 175) (Cell Signaling

Technology, USA). The same immunohistochemistry procedure was performed for all antibodies as described previously (Danilović Luković et al., 2017). Rabbit Specific HRP/DAB (ABC) Detection IHC Kit (ab64261, Abcam, UK), was used for reaction visualization.

Semiquantitative assessment of PCNA and caspase-3 expression

PCNA and caspase-3 expression in the OSE cells was assessed on all parts of OSE per section. To obtain data indicating the expression of antigen or enzyme within cells, staining intensity was reported as negative (-), moderate (+) or strong (++) positive. The number of the particular cell types was given as a percentage, relative to the total number of counted cells (150) in each examined section.

Histological and morphometric analyses were conducted using the microscope Olympus CX43 equipped with Olympus Digital Camera C7070 and Olympus Cell Sens software.

Statistical analysis

Morphometric and semi-quantitative analysis results are expressed as mean ± standard error. All values above and below the confidence interval (5-95%) were considered irrelevant and were excluded from further processing of results. Student's t-test was used to determine statistical differences between groups. Levels of significance were: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

RESULTS

Histological analysis of OSE is shown in the Figure 1. No major structural differences were found comparing the OSE between the control and the hypothyroid groups at the given ages, except for the appearance of the apical part of the OSE cells in the hypothyroid newborn and neonatal rats. The apical cell membrane of the OSE cells in the ovaries of these rats appears to be incomplete, with pronounced cytoplasmic projections (Figure 1).

In short, ovaries of both hypothyroid and control newborn rats showed that OSE was pseudostratified and that it mostly consisted of cuboidal and/or columnar cells, without a clearly differentiated basal membrane. Nuclei were pleomorphic and located mainly in the apical part of the cell. Tunica albuginea was not observed in newborn rats, but occasionally, groups of fibroblast-like cells were present below the OSE cells.

In the ovary of neonatal rats, the OSE was single-layered, mainly composed of cuboidal and occasionally squamous cells, positioned in the clearly visible basement membrane. The mitotic activity of these cells was sporadic. Pleomorphic nuclei had a central or, less frequently, an apical localization in the OSE cells. Tunica albuginea was clearly observed, consisted of two or more layers of fibroblast-like cells.

In both newborn and neonatal rats' ovaries, in the control and the hypothyroid groups, oocyte-like cells were noticed between or, sporadically, immediately below the OSE cells (Figure 1).

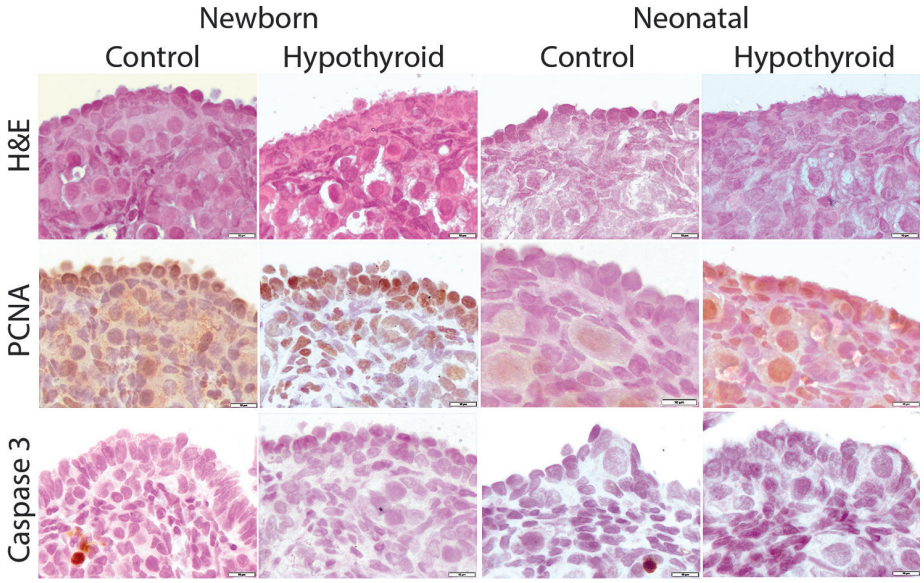

Figure 1. Sections through the ovary of the controlled and the hypothyroid newborn and neonatal rats. Hematoxylin/eosin (H&E); proliferating cell nuclear antigen (PCNA), and caspase-3 expression in ovaries of control and hypothyroid newborn and neonatal rats. Bar 10 μm.

OSE cell height

Our results concerning OSE height showed a significant difference ($p<0.001$) when the newborn and neonatal hypothyroid pups were compared as well as when the comparison was made among the control group and hypothyroid ones within the neonatal age group (Figure 2). No significant difference was found between the control and hypothyroid groups within the newborn rats, or in the control group of newborn rats compared to the control group of neonatal rats (Figure 2).

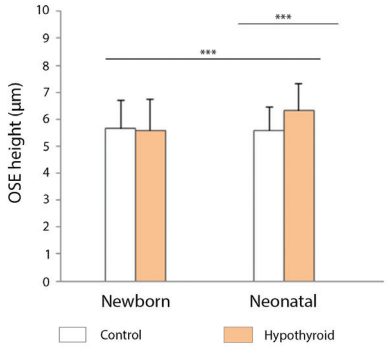

Figure 2. Ovarian surface epithelium height of newborn and neonatal control and hypothyroid rats. Level of significance: ***$p<0.001$

Results regarding the PCNA and the caspase-3 immunostaining are presented in Figure 1 and Table 1. PCNA was localized in the nucleus and the perinuclear area of the OSE cells. Histological analysis showed that both newborn and neonatal hypothyroid rats had greater percentages of, and more intensively marked, PCNA-positive OSE cells than the corresponding controls. The percentage of strongly PCNA immunopositive cells was 3.21 times higher in hypothyroid newborn rats than in the control group. Also, in the neonatal period, the percentage of PCNA immunopositive cells was 3.08 and 7.29 times higher (moderately and strongly positive cells, respectively) in the OSE of hypothyroid rats (Table 1).

On the other hand, most of the OSE cells in both ages and groups were caspase-3 negative (Table 1). Cleaved caspase-3 immunopositive cells were rare in OSE of neonatal and newborn rats from both groups, but when positive, it was localized in the nucleus or in the perinuclear area (data not shown).

Table 1. Percentages of proliferating cell nuclear antigen (PCNA) and caspase-3 immunopositive cells in the ovarian surface epithelium of newborn and neonatal control and hypothyroid rats.

		PCNA (%)			Caspase-3 (%)		
		–	+	++	–	+	++
Newborn	Control	22.08	61.69	16.23	100	0	0
	Hypothyroid	16.09	31.83	52.08	99.55	0.45	0
Neonatal	Control	84.73	11.45	3.82	91.12	2.88	2
	Hypothyroid	36.82	35.82	27.86	100	0	0

DISCUSSION

Expression of PCNA was significantly increased, but no alterations in caspase-3 dependent apoptosis were recorded in the OSE cells of newborn and neonatal rat ovaries as a consequence of maternal hypothyroidism. Additionally, a slight increase in the OSE height and apical membrane disruption was present.

No major differences due to maternal hypothyroidism were noticed on the light microscopic level as far as the OSE morphology was concerned. This result was not unexpected, as our previous results have shown that hypothyroidism induces changes in ovarian tissue that can be visualized only on the ultrastructural level (Danilović Luković et al., 2016). The typical changes were described as enlarged smooth endoplasmic reticulum (SER) (Danilović Luković et al., 2016). Therefore, our assumption is that numerous enlarged SER could contribute to the augmentation of the cytoplasm volume and consequently the OSE cell height. Likewise, the apparent morphological transition of OSE from pseudostratified to single-layered could be the response to ovarian surface enlargement, which itself requires a change in epithelial cells volume. In other words, cells are "stretching" from columnar to cuboidal or, in some places, squamous shape. Single-layered OSE appears after primordial follicle

pool formation in humans and rodents and is a characteristic of adult ovary (Gondos, 1975; Papadak & Beilby, 1971). In other types of epithelial cells, the increase of the cell height indicated alteration of cell differentiation or increased proliferation rate (Wu et al., 2007). The incompleteness of the apical membrane of the OSE cells seen in our hypothyroid rat group advocates in favor of altered cell differentiation. Reduced levels of TH, which are necessary for the maintenance of proper cell proliferation and differentiation (Nicholson & Altman, 1972), could lead to the increase of OSE height recorded in neonatal rats in our study. Further analysis of apical plasma membrane glycoprotein or other markers (Mostov et al., 1992) and the ultrastructure of these cells could be a step forward in the better understanding of the negative effects of lack of TH on OSE cells.

Intensive tissue remodeling occurs during early postnatal period in rats and is characterized by intensive cell proliferation and pronounced expression of PCNA in ovaries (Xu et al., 2011), which is enhanced in hypothyroid rats. On the other hand, uncontrolled proliferation underlies cancer development (López-Sáez et al., 1998), so we suggest that ovaries in this period can serve as a good model for understanding carcinogenesis in OSE (Caric et al., 2014). Enhanced PCNA immunoexpression also implies intensive DNA repair in hypothyroid rats of both ages in our study, and could be explained as an attempt of survival of healthy or slightly damaged cells (Xu et al., 2011) but also could indicate introduction of the cells into apoptosis or autophagy (Danilović Luković et al., 2017). Although caspase-3 apoptosis is engaged in somatic but not germ cell elimination (Matikainen et al., 2001), no detected expression of caspase-3 in OSE cells of both ages and groups in our study leads to the conclusion that this pathway may not be responsible for the removal of OSE cells in the early postnatal period. It is also known that autophagy is a dominant means of germ cell elimination in the ovary in this period (Danilović Luković et al., 2017). It would be interesting to further examine the types of cell death involved in the process of adult OSE formation.

CONCLUSION

Our results indicate that hypothyroidism enhances the proliferation of OSE and the absence of caspase-3-dependent apoptosis. Further investigation should clarify the mechanisms of OSE cell removal in the postnatal period and any possible link between the intensive proliferative activity of OSE cells and potential carcinogenesis. Currently, OSE is still an enigma in terms of developmental biology and prevention and treatment of ovarian cancer.

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Authors contributions

AR and JDL conceived defined a research theme. JDL, IM and TL carried out animal welfare, immunohistochemical studies and statistical analysis. VS acqures new literature data and with AJ done the experimental part of the study including histomorphometric analyses. JDL and VS were writing the initial text. MKF and AR were involved in drafting the manuscript and revising it critically for important intellectual content and have made a substantial contribution to conception and design, analysis and interpretation of data. All authors discussed the results and contributed to the final manuscript.

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POVRŠINSKI EPITEL JAJNIKA HIPOTIREOIDNIH NOVOROĐENIH I NEONATALNIH PACOVA: IZ PERSPEKTIVE PCNA I KASPAZE-3

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Kratak sadržaj

Uvod. Tokom reproduktivnog perioda, nakon svake ovulacije, površinski epitel (PE) jajnika se intenzivno regeneriše i remodelira. Ovaj proces povećava rizik od nastajanja jednog od najčešćih tumora jajnika kod žena i kuja. Imajući u vidu činjenicu da hipotireoidizam majki u velikoj meri utiče na proliferaciju i ćelijsku smrt u toku folikulogeneze u ranom postnatalnom periodu, cilj rada je bio da analiziramo efekat hipotireoidizma majki na morfologiju i dinamiku PE jajnika potomaka.

Materijal i metode. U eksperimentu su korišćeni tek rođeni (24 časa stari) i neonatalni (4 dana stari) mladunci koji potiču od kontrolnih i hipotireoidnih majki. Morfometrijskom analizom određivana je prosečna visina ćelija PE, izmerena na četiri mesta na svakom ispitivanom preseku jajnika. Takođe, imunopozitivnost proliferativnivnog ćelijskog nuklearnog antigena (PCNA) i kaspaze 3 je određivana semikvantitativno.

Rezultati i zaključi. Veće promene u strukturi PE jajnika nisu zapažene između grupa, osim blagog povećanja visine ćelija i nepotpune apikalne membrane sa projekcijama citoplazme kod hipotireoidnih životinja. Imunopozitivnost PCNA je značajno povećana u jajnicima hipotireoidnih životinja obe starosne grupe u odnosu na kontrole, dok je imunopozitivnost na kaspazu 3 slabo izražena kod svih životinja. Naše istraživanje je pokazalo da hipotireoidizam u ranom postnatalnom periodu dovodi do promena u morfologiji i proliferaciji ćelija PE, bez efekta na uklanjanje ćelija delovanjem kaspaze 3. Ovaj model može da posluži u budućim istraživanjima potencijalne karcinogeneze u cilju prevencije i tretmana kancera jajnika.

Ključne reči: Apoptoza, hipotireoidizam majki, proliferacija