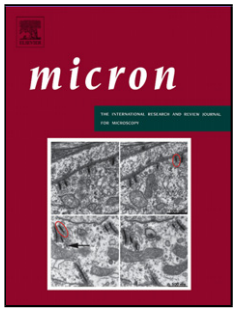

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Altered state of primordial follicles in neonatal and early infantile rats due to maternal hypothyroidism: light and electron microscopy approach

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Highlights

Hypothyroidism accelerated formation of primordial follicles

Primordial follicle oocytes shows the significant alterations of sER

Primordial follicle oocyte had increased amount of mitochondria with altered morphology

Advanced follicle maturation increases apoptotic and proliferating markers expression

Abstract

Thyroid hormones (TH) are one of the key factors for normal prenatal development in mammals. Previously, we showed that subclinical maternal hypothyroidism leads to premature atresia of ovarian follicles in female rat offspring in the pre-pubertal and pubertal periods. The influence of decreased concentration of TH on primordial follicles pool formation during neonatal and early infantile period of rat pups was not investigated previously. Maternal hypothyroidism during pregnancy has irreversible negative influence on primordial follicles pool formation and population of resting oocytes in female rat offspring. The study was done on neonatal and early infantile control (n-10) and hypothyroid (n-10) female rat pups derived from control (n-6) and propylthiouracil (PTU) treated pregnant dams (n-6), respectively. Ovaries of all pups were removed and processed for light and transmission electron microscopy (TEM). Number of nests, oogonia and oocytes per nest, primordial, primary, secondary and preantral follicles were determined. Screening for overall calcium presence in ovarian tissue was done using Alizarin red

staining. Morphology and volume density of nucleus, mitochondria and smooth endoplasmic reticulum (sER) in the oocytes in primordial follicles was also assessed. Caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), both markers for apoptosis, and proliferating cell nuclear antigen (PCNA) for proliferation were determined in oocytes and granulosa cells in different type of follicles. In neonatal period, ovaries of hypothyroid pups had a decreased number of oogonia, oocytes and nests, an increased number of primordial follicles and a decreased number of primary and secondary follicles, while in early infantile period, increased number of primary, secondary and preantral follicles were found. Alizarin red staining was intense in hypothyroid neonatal rats that also had the highest content of dilated sER. Number of mitochondria with altered morphology in both groups of hypothyroid pups was increased. Apoptosis markers have not shown significant difference between groups but PCNA had an increased expression in the oocytes and granulosa cells in primordial follicles of hypothyroid rats. Light and electron microscopy analysis indicate that previously detected premature ovarian follicular atresia in pre-pubertal and pubertal hypothyroid rats is preceded with premature formation of primordial follicles followed by slight changes on sER and mitochondria in examined oocytes, and increased expression of PCNA.

Keywords: Hypothyroidism; primordial follicle; PCNA; Caspase-3; TUNEL; oocyte ultrastructure;

1. Introduction

Hypothyroidism induces alterations of ovarian function. It is known that transient hypothyroidism in immature rats induces polycystic ovary-like syndrome (Bagavandoss et al., 1998), atrophy and underweight of ovaries in adult rats (Ortega et al., 1990) and atresia of follicles (Meng et al. 2016) while even subclinical hypothyroidism in women induces infertility (Abalovich et al 2007). In developed countries, the prevalence of subclinical hypothyroidism in child-bearing age may be as high as 5% (review: Gharib et al., 2005). Although it is recognised that hypothyroid woman could have full-term carriage, foetal development may be abnormal (De Groota et. al., 2012). The most important consequence of hypothyroidism during pregnancy is irreversible suboptimal brain development of the child as thyroid hormones (TH) are necessary for early neuronal development (Haddow et al., 1999). We hypothesised the same should be true for fertile

oocyte formation in female child. The argument behind this hypothesis relies on a fact that the pool of oocyte precursors, i.e. resting oocytes in primordial follicles, is formed during prenatal development in humans (Fulton et. al 2005). Thus, hypothetically, the oocytes in female child of pregnant hypothyroid women, could acquire irreversible changes during prenatal development, that could be visible only in the moment when they start to be activated, i.e. in pre-pubertal or pubertal period. Previously, using propylthiouracil (PTU) treated pregnant and lactating dams as a model for maternal hypothyroidism, we were able to show that their female offspring had an early activation of follicles followed by their atresia in late infantile, pre-pubertal and pubertal period (Radovanović et al., 2012). Reduction in the number of follicles and corpora lutea, and hyperplasia and hypertrophy of stromal interstitial cells also implied premature ovarian failure in the ovaries of pubertal rats (Radovanović et al., 2012).

TH act on nuclear and membrane receptors regulating overall metabolic pathways and cell proliferation and differentiation in peripheral tissues, but they also orchestrate body functions acting through central nervous system (Lopez et al., 2013). Depending on tissue and cell type, as well as severity and duration of hypothyroidism, cell cycle could be activated or inhibited and apoptogenic signals could be induced (Holsberger et al., 2013; Alisi et al., 2005). Previous works on developing rat ovaries showed that neonatal hypothyroidism alters differentiation, but not proliferation of granulosa cells (Djikstra et al., 1996). Increased apoptosis of granulosa cells, without oocyte alteration in pre-pubertal rats was detected when postnatal hypothyroidism was induced (Chan and Ng, 1995). Although several papers describe the effect of hypothyroidism on folliculogenesis, no data exists for the neonatal and early infantile period of rat pup development i.e., the most important period for resting oocytes pool formation and subsequent reproductive potential.

Apart from acting to nuclear receptors, TH act directly on several organelles. TH action upon mitochondria function is well described, while their action upon endoplasmic reticulum (ER) is still not explored. Most of the data demonstrating TH effect on mitochondria were retrieved from experiments on rat hepatocytes and different type of muscle cells *in vivo*, or isolated cells and organs (review: Harper and Seifert, 2008). Namely, decreased TH concentration is connected with lower numbers of mitochondria per cell and decreased ATP regeneration, basal proton leak, and oxygen consumption (review: Harper and Seifert, 2008). In hypothyroidism, mitochondrial membranes are more fluid due to altered fatty acyl composition (Brookes et al, 1998), probably

influencing their morphology. Alteration and interdependence of mitochondrial morphology and function have been shown in different metabolic disorders: diabetic cardiomyopathy on cardiomyocytes and in non-alcoholic fatty liver disease on hepatocytes (review: Galloway and Yoon, 2012). Of importance, it was shown that hypothyroidism also altered mitochondrial morphology and led to the release of apoptogenic proteins during rat cerebellar development (Singh et al., 2003). Although mitochondria are essential for oocytes metabolism in resting primordial follicles (review: Sutton-McDowell et al., 2010), no data about their morphology in hypothyroid animals exists.

TH binding protein (p55) and deiodinases located on endoplasmic reticulum (ER) are important regulators of TH biological activity (Cheng et al, 1987, Köhrle, 1995). ER is involved in biosynthesis of proteins, phospholipids and cholesterol and degradation of glycogen. Orchestrated action of ER and mitochondria is important for Ca^{++} homeostasis and apoptosis (for review see: Bauman & Waltz, 2001; Berridge, 2002). It is interesting that TH regulate Ca^{++}-ATPases pumps on cell membranes and endoplasmic reticulum via non-genomic mechanisms (Incerpi et al, 2008). The influence of decreased concentration of TH on ER is not known, but one could speculate that lack of TH would alter Ca^{++} flow from ER and alter different signalling cascades ultimately leading to apoptosis.

Using a model of maternal hypothyroidism in rats to mimic hypothyroid state of a pregnant woman, we tested the effect of TH deficiency on ovarian development in the neonatal and early infantile periods of female rats. The aims of the present study were 1) to investigate if premature follicular atresia seen in the juvenile and pubertal periods is preceded with alteration in primordial follicles assembly and their activation into primary follicles, 2) to examine subcellular changes in primordial follicles, emphasising mitochondria and ER as organelles important for TH non-genomic action and 3) to examine if hypothyroidism alters proliferation of granulosa cells and apoptosis of granulosa cells and oocytes.

2. Materials and Methods

2.1. 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 12h 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 randomised 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 neonatal (four-day-old) and five early infantile (seven-day-old) female pups from treated mothers and another ten matched by age from control mothers were euthanized using a prolonged deep anaesthesia and their body weights were recorded. Blood sampling was carried out during the anaesthesia protocol. Concentration of triiodothyronine (T3) and thyroxine (T4) was determined using radioimmunoassay kits (INEP, Zemun, Serbia). The experiment 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 EU and OECD. All experimental procedures were performed under the supervision of a licensed veterinarian, who specialised in the conduction of experiments on laboratory animals. All researchers and technicians who were part of the experiment are authorised to perform experiments in laboratory animals.

2.2. Tissue processing and sampling for light microscopy (histochemistry and stereology)

The ovaries and thyroid glands were removed and fixed in 10% neutral-buffered formalin (pH 6.8) 24 hours at room temperature. To ensure uniform sagittal sections, dehydrated ovaries were embedded in paraffin with the longest axis face down. As ovaries contained oogonia, and oocytes in different stages of first meiotic prophase, it was estimated that the best way to analyse them is the previously established method for tissues with high cell variability. Namely, each fifth section (5 µm thick) was used for morphometry analysis (Bucci et.al., 1997, Tilly, 2003, Picut et. al., 2015). The approximate number of sections per ovary was between 20 and 25. Number of nests in ovarian cortex and average number of oogonia/oocytes per nest were assessed in follicles and the number of primordial, primary, secondary and preantral follicles (modified according to

Mazaud et al, 2002) was given as a number of follicles per section. Primordial follicle is defined as an oocyte partially or completely surrounded with flattened granulosa cells, primary follicle as an oocyte with one layer of cuboidal granulosa cells or at least one cuboidal cell among flattened granulosa cells, secondary follicle as an oocyte with two layers and more of cuboidal granulosa cells or one layer and at least one cell of second layer and preantral as an oocyte surrounded with more than two layers of granulosa cells and Call-Exner bodies between them (see supplementary data 1). Although, it was recently published that using optical disector could be an accurate method for quantification of oocytes with visible nucleoli in pubertal and adult ovaries (Charleston et al, 2007; Bordbar et al, 2014), rare nucleoli and oogonia/oocytes variability in developing rat ovaries made disector analysis less desirable in conditions described in this experiment.

2.3. Alizarin red staining for Ca^{++} detection

Staining for Ca^{++} was performed in Alizarin Red Solution (C.I. 58005, 2 gm in 100 ml of distilled water). The pH was adjusted to 4.1–4.3 with 10% ammonium hydroxide. Slides were held in dye for 1 minute. Dehydration proceeded in acetone (100%) for 20 dips, then in Acetone-Xylene (1:1) solution for 20 dips. Slides were cleared in xylene and mounted in a DPX mounting media.

2.4. Hypothyroidism determination

To determine hypothyroidism, thyroid gland stereology and activation index value was assessed. Thyroid gland was analysed using a multipurpose stereological grid M42 (The M42 testing system had 21 straight-line segments and 42 testing points in a testing area equal to 36.36 d2). The volume density of different thyroid compartments was determined by using model - based stereology (Weibel, 1979). First, volume density (Vv) of interstitium (Vvi), epithelial tissue (Vve) and colloid (Vvc) were determined. Using stereological analyses, the activation index (Ia) of thyroid gland was calculated by means of the following formula:

$$Ia = Vve / Vvc$$

2.5. Tissue processing for electron microscopy

Ovaries were fixed in 4% glutaraldehyde for 24 hours (pH 7.2), rinsed in the Milloning buffer three times and post-fixed in 1% osmium tetroxide (OsO4, pH 7.2) for 1 h. After dehydration in a graded series of acetone, the tissue was embedded in araldite. Semi-thin sections were stained with Toluidine blue and used to select areas of interest. Ultra-thin sections of selected areas were obtained using Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany), mounted on copper grids and contrasted in uranyl acetate and lead citrate using Leica EM STAIN (Leica Microsystems). Sections were examined on a Philips CM12 transmission electron microscope (Philips/FEI, Eindhoven,Netherlands) equipped with the digital camera SIS MegaView III (Olympus Soft Imaging Solutions, Münster, Germany).

2.6. TEM stereology

Stereology-based quantitative characterisation of cytoplasm and cellular compartments (mitochondria, smooth endoplasmic reticulum - sER, Golgi complex and nucleus) of oocytes of primordial follicles was performed on TEM photographs using a Adobe Photoshop CS6 Software. The stereological analysis was performed only on the oocytes with the nuclear mid-section, at a low (× 3000) magnification. Random test points were assigned on microphotographs and volume density of examined cytoplasm and cellular compartments were given according to formula $Vv = P_c/P_{total}$, where P_c is the number of points on a certain cell portion (cytoplasm, organelle) and P_{total} is a number of points in the frame (Weibel et al, 1966).

2.7. Mitochondria number and area determination

To determine fraction of each mitochondrial type in total mitochondrial number per oocyte section and area of each type of mitochondria (M1, M2 and M3, described below in section Results), randomly selected primordial follicles from electron micrographs, taken at magnification x 3000, were used. All mitochondria in the cytoplasm of selected oocytes were analysed, with no less than 100 mitochondria of each type.

2.8. Immunohistochemistry

Paraffin embedded tissue sections were used to determine the PCNA (proliferating cell nuclear antigen) (sc-7907) (Santa Cruz, Biotech, USA), cleaved caspase-3 (Asp 175) (Cell Signaling Technology, USA) and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) (FragEL DNA fragmentation Detection kit with colorimetric TdT enzyme (QIA33, Merck) localisation. For all others, except TUNEL, the following procedure was performed: slides were microwaved in citrate-buffer 0.1M (pH 6) for antigen retrieval. After cooling at room temperature and washing twice in phosphate-buffered saline (PBS), blocking of endogenous peroxidase was performed with Peroxidase Block System for 10 minutes. Then, tissue was washed in PBS and blocked with Protein Block for 5 minutes (Novocastra Peroxidase Detection System, Leica, UK). Slides were washed two times in PBS and incubated with anti-PCNA (1:100) and anti-caspase-3 antibody (1:100), diluted in 5% Normal Goat Serum, overnight at 4ºC. On the negative controls, primary antibody was not used. After washing, slides were incubated at room temperature with secondary antibody (30 minutes), with streptavidin-HRP (30 minutes) and then washed. Reaction was developed with 3-3'-diaminobenzidine (DAB) (Novocastra Peroxidase Detection System, Leica, UK). Counterstaining was carried out with Mayer's haematoxylin and slides were mounted with DPX. For TUNEL detection, we followed manufacture's protocol (Merck Millipore, Darmstadt, Germany).

PCNA, caspase-3 and TUNEL staining index of ovarian follicles was calculated according to the formula previously used by Moggadham-Dorafshani et al., (2013):

$$A \times B \times 100 / C \times D$$

A=number of follicles with positive cells; B=number of positive cells in each follicle; C=total number of follicles; D=total number of cells in each follicle

To obtain data indicating caspase-3 and TUNEL expression in the oocytes, only follicles with positive cells were counted, i.e., follicles from previous formula that were labelled with A. Staining intensity was reported as negative (-), weak (+), moderate (++) and strong (+++) positive.

To obtain data indicating PCNA expression in the primordial follicles oocytes, the number of positive oocytes was expressed as a percentage of total number of oocytes.

Histological and morphometric analyses were done using microscope Olympus CX43 with Olympus Digital Camera C7070.

2.9. Statistical analysis

Counting and all stereological analyses were performed by two independent researchers who observed all slides two times for morphological and stereology analysis and three times for immunohistochemical evaluations. All values above and below the confidence interval (5-95%) were considered irrelevant and excluded from further processing of results.

Stereology, morphometry and semi-quantitative analysis results are expressed as mean± standard error. Student's t-test was used to determine statistical differences between groups. Levels of significance were: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

3. Results

3.1. Treated neonatal and early infantile rats had decreased body mass and were hypothyroid

Neonatal hypothyroid pups had, on average, 24% lower body mass compared to the controls. The difference in body weight between pups in two groups was less evident in the early infantile period and was, on average, 16% (see supplementary data 2).

Thyroid glands in neonatal and early infantile pups from treated mothers had abundant micro-follicles, with columnar epithelium and small amount of colloid with frequent mitosis (see supplementary data 1). Thyroid activation index was 25% increased and T3 and T4 concentrations were 25% decreased in both neonatal and early infantile hypothyroid rats when compared to control animals (see supplementary data 2).

3.2. Hypothyroidism accelerated formation of primordial follicles

Ovaries of neonatal and early infantile pups in both groups had no clear boundaries between cortex and medulla and the follicles were distributed in the entire ovary (Figure 1-ABCD). Under the surface epithelium and sparse tunica albuginea in the ovaries of both groups in the neonatal period, nests containing groups of resting and dividing oogonia and oocytes and primary and secondary follicles were present (Figure 1-A and B). In the early infantile period, nests were not present, but primordial, primary, secondary, and preantral follicles could be seen in both groups (Figure 1-C and D). Ovaries of neonatal hypothyroid pups had a decreased number of nests and decreased number of oogonia and oocytes per nest, as well as increased number of primordial follicles and decreased number of primary and secondary follicles ($p<0.05$) (Figure 1-A and B, Table 1). In the early infantile period, nests were not present. The number of primordial follicles was almost equal between groups, but primary, secondary, and preantral follicles dominated in ovaries of hypothyroid pups (Figure 1-C and D, Table 1).

Preantral follicles were not present in ovaries of neonatal rats (Figure 1-A and B, Table 1). Numbers of primordial and secondary follicles were significantly higher in ovaries of early infantile hypothyroid rats in comparison to neonatal hypothyroid rats (Table 1). In ovaries of control animals, the number of primordial follicles was significantly higher, and the number of

primary follicles was significantly lower in early infantile pups compared to neonatal pups (Table 1).

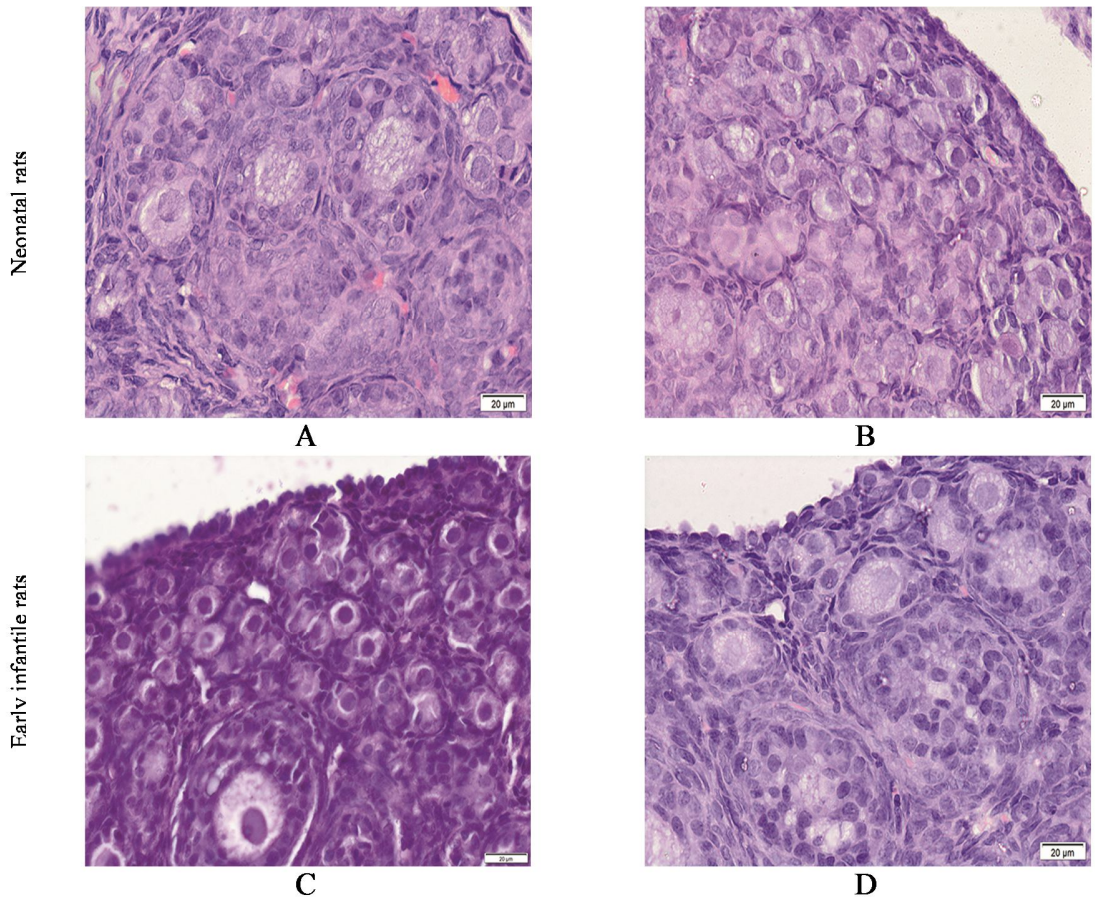

Figure 1. Section through of the ovary of neonatal control (A) and hypothyroid (B) and early infantile control (C) and hypothyroid (D) rats. H&E. Bar 20 µm.

Type of follicle	Neonatal		Early infantile	
	Control	Hypothyroid	Control	Hypothyroid
Nests	4.98 ± 1.39	2.85 #,** ± 1.39	0	0
Oogonia and oocytes per nest	5.74 ± 0.99	4.62 #,* ± 0.69	0	0
Primordial	31.56 ± 0.46	64.69 #,*** ± 2.18	57.9 #,*** ± 5.99	54.27 ¤,*** ± 3.92
Primary	48.36 ± 5.16	40.76 #,* ± 5.53	27.12 #,*** ± 4.75	35.32 §,* ± 5.55
Secondary	26.29 ± 3.55	21.72 #,* ± 2.95	26.82 ± 5.46	31.68 ¤,** ± 6.4
Preantral	0	0	2.92 ± 0.74	5.77 §,* ± 1.36

Table 1. Comparative review of number of nests, oogonia and oocytes per nest, primordial, primary, secondary and preantral follicles per ovary section of control groups of neonatal and early infantile days old rats. Results were presented as mean ± SE. Levels of significance: *p<0.05; **p<0.01; ***p<0.001. # - versus neonatal control, § - versus early infantile control, ¤ - versus neonatal hypothyroid.

3.3. Primordial follicle oocytes in neonatal hypothyroid rats show the significant dilatation of sER

The intensity of Alizarin Red staining was higher in neonatal rats when compared to early infantile period in both groups (Figure 2-A1 and A2). This staining demonstrated higher level of Ca^{++} in oocytes of hypothyroid rats, which indicated the possible changes in ultrastructural level. Subsequent ultrastructural analysis of primordial follicles, in neonatal and early infantile rats in both groups of animals, demonstrated the presence of oocytes and granulosa cells with finely dispersed chromatin in nuclei with eccentric one or two nucleoli (Figure 2-BCDE). Oocytes of hypothyroid neonatal rats had dilated sER when compared to the controls (Figure 2-B and C). Oocyte cytoplasm analysed at higher magnification shows different appearance of dilated and normal sER (Figure 2-F1 and F2). In the early infantile period there was no difference in sER morphology between two groups (Figure 2-D and E). Randomly dispersed vesicular cisternae of sER characterised oocyte cytoplasm of hypothyroid neonatal rats (Figure 2-C). On the contrary, dilatations of endoplasmic reticulum, if present, were small and rare in both groups of early infantile rats (Figure 2-D and E).

Stereology-based analysis of oocytes in primordial follicles in neonatal and early infantile rats showed that the hypothyroid group had higher volume density of nucleus and sER and lower volume density of cytoplasm (Table 2). In hypothyroid animals, volume density of sER in the oocytes was higher in neonatal when compared to early infantile rats (Table 2). Volume density of Golgi complex and mitochondria was not different between groups, although a slight increase was detected in both neonatal and hypothyroid animals when compared to the controls (Table 2).

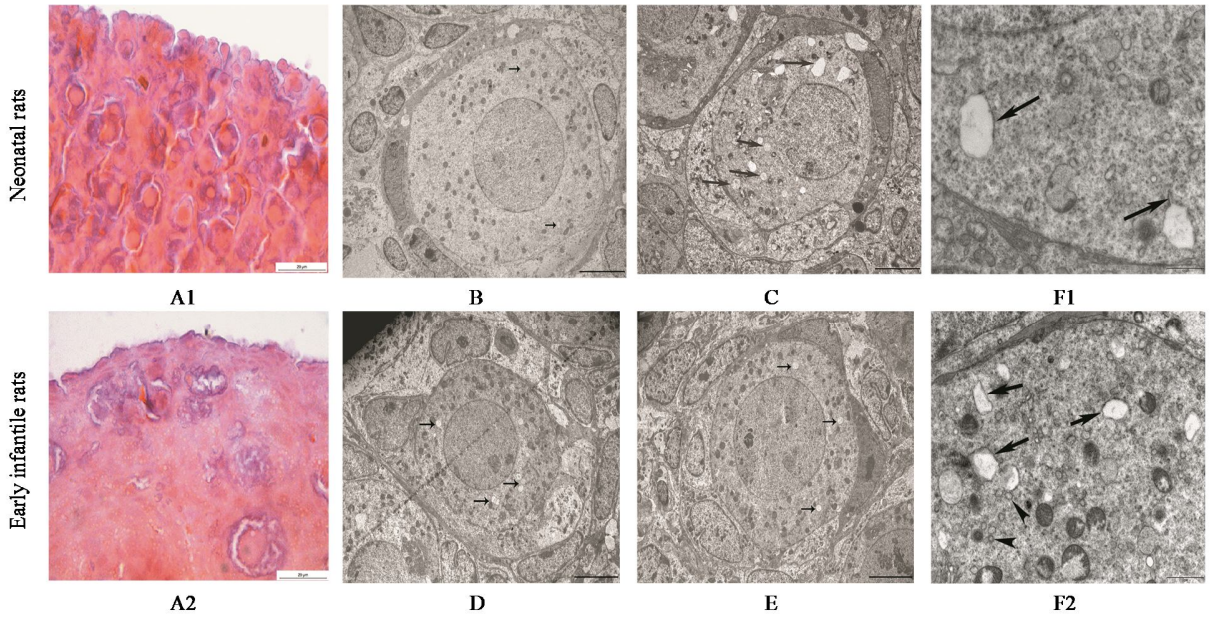

Figure 2. Alizarin red staining of ovaries of neonatal (A1) and early infantile (A2) rats. Primordial follicles TEM of neonatal ovaries in control (B) and hypothyroid (C) rats. Primordial follicles TEM of early infantile ovaries in control (D) and hypothyroid (E) rats. Arrows pointing the vacuolization in cytoplasm. Bar 20 μm for light microscopy, 5 μm for TEM. Dilated profiles of sER (F1 and F2). Note the 'small tail' on dilated sER (arrows), indicating it's continuity with existing tubule-reticular domain (F1). Several dilated (arrows) and normal (head arrows) sER profiles (F2). Bar 1 μm.

Cell compartment	Neonatal		Early infantile	
	Control	Hypothyroid	Control	Hypothyroid
Nucleus Vv (%)	22.41 ± 12.11	30.67 #,* ± 6.23	30.1 ± 7.22	28.53 ± 8.02
Cytoplasm Vv (%)	61.23 ± 11.71	47.44 #,** ± 4.3	52.77 ± 5.8	54.04 ¤,* ± 8.03
Mitochondria Vv (%)	7.3 ± 1.27	8.04 ± 1.96	7.61 ± 3.02	8.03 ± 2.28
Smooth endoplasmic reticulum Vv (%)	7.06 ± 1.95	10.02 #,* ± 3.51	8.06 ± 1.93	5.56 §,**, ¤,*** ± 1.08
Golgi complex Vv (%)	2 ± 1.33	3.83 ± 2.89	1.46 ± 1.75	3.84 ± 3.74

Table 2. Volume density (Vv) of different cell compartments in oocytes of primordial follicles of control and hypothyroid neonatal and early infantile rats. Results were presented as a percentage (mean ± SE). *p<0.05; **p<0.01; ***p<0.001. # - versus neonatal control, § - versus early infantile control, ¤ - versus neonatal hypothyroid.

3.4. Primordial follicle oocytes contained increased number of mitochondria with altered morphology

Mitochondria were evenly distributed in the cytoplasm of all tested groups and periods. In all conditions examined, three types of mitochondria were defined based on their morphology:

type one (M1) with elongated dark cristae and pale matrix, type two (M2) with short cristae and wide pale central area and type three mitochondria (M3) with discontinued membrane and reduced number of cristae (Figure 3-A). Oocytes in neonatal hypothyroid rats had both increased number of and area occupied with M2 and M3 mitochondria (Figure 3-B and C). Oocytes in early infantile hypothyroid rats had decreased number of M1 and increased number of M3 mitochondria, i.e., those with discontinued membrane (Figure 3-D). Area occupied with M2 mitochondria was significantly increased in hypothyroid rats in the early infantile period (Figure 3-E).

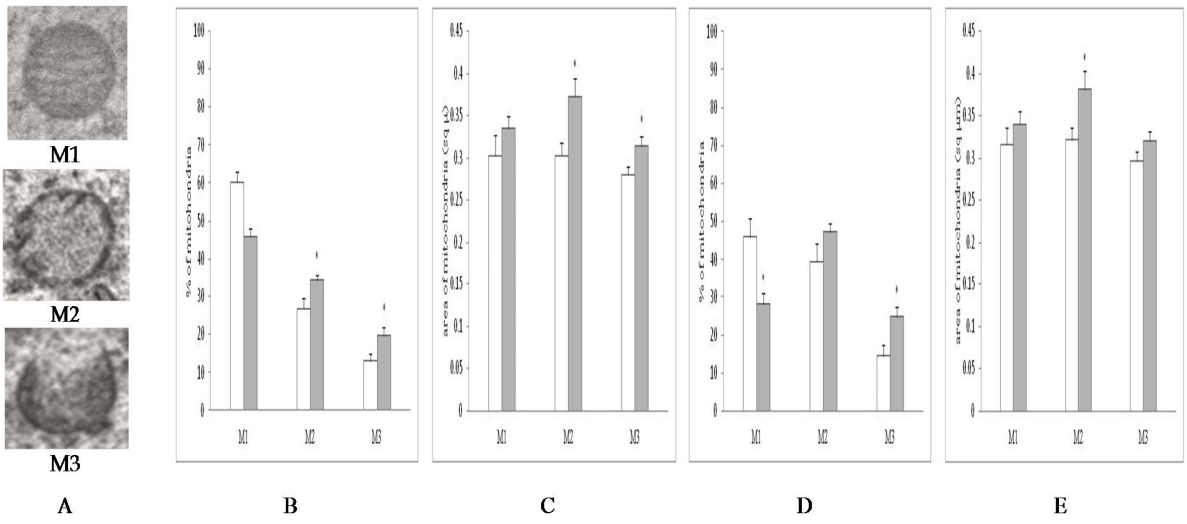

Figure 3. Three types of mitochondria (M1, M2, M3) (A). Percentage of three types of mitochondria in neonatal (B) and early infantile (D) rats. Average area of three types of mitochondria in neonatal (C) and early infantile (E) rats. Open bars, control group; gray bars, hypothyroid. Results are presented as mean ± standard error (M ± SE). Level of significance: * $p<0,05$.

3.5. Advanced maturation of follicles is associated with higher expression of caspase-3, TUNEL and PCNA staining

Cleaved caspase-3, when positive, was localised in nucleus and in perinuclear area of oocytes and granulosa cells in neonatal period from both groups (Figure 4-A and B). Oocytes forming primordial follicles in neonatal rats from both groups did not stain for cleaved caspase-3

(Figure 4-C). Oocytes forming primary follicles in control group in neonatal period were caspase-3 negative, while in hypothyroid animals oocytes were slightly positive (Figure 4-C). In secondary follicles, oocytes from both groups had moderate positive staining for caspase-3 (Figure 4-C). In neonatal period, granulosa cells in primordial follicles were caspase-3 negative, while in primary and secondary follicles about one-third and about one-half were caspase-3 positive in both groups (Figure 4-D). TUNEL signal was localised in nucleus of oocytes and granulosa cells in neonatal period (Figure 4-E and F). TUNEL positive pre-diplotene oogonia or/and oocytes were noticed in both groups (Figure 4-E and F). In neonatal period, oocytes and granulosa cells in primordial follicles of control and hypothyroid group were TUNEL negative (Figure 4-G and H). Oocytes in primary follicles of both groups expressed weak signal (Figure 4-G). Oocytes in secondary follicles of control group were weakly stained, while those in hypothyroid group were moderately stained (Figure 4-G). In primary follicles, about one-third and in secondary follicles about one-half of granulosa cells were TUNEL positive in both groups (Figure 4-H). PCNA was localised in the nucleus and perinuclear area of oocytes and granulosa cells in both groups of animals (Figure 4-I and J). In neonatal period, hypothyroid pups had more PCNA positive oocytes and granulosa cells in all types of follicles (Figure 4-K and L).

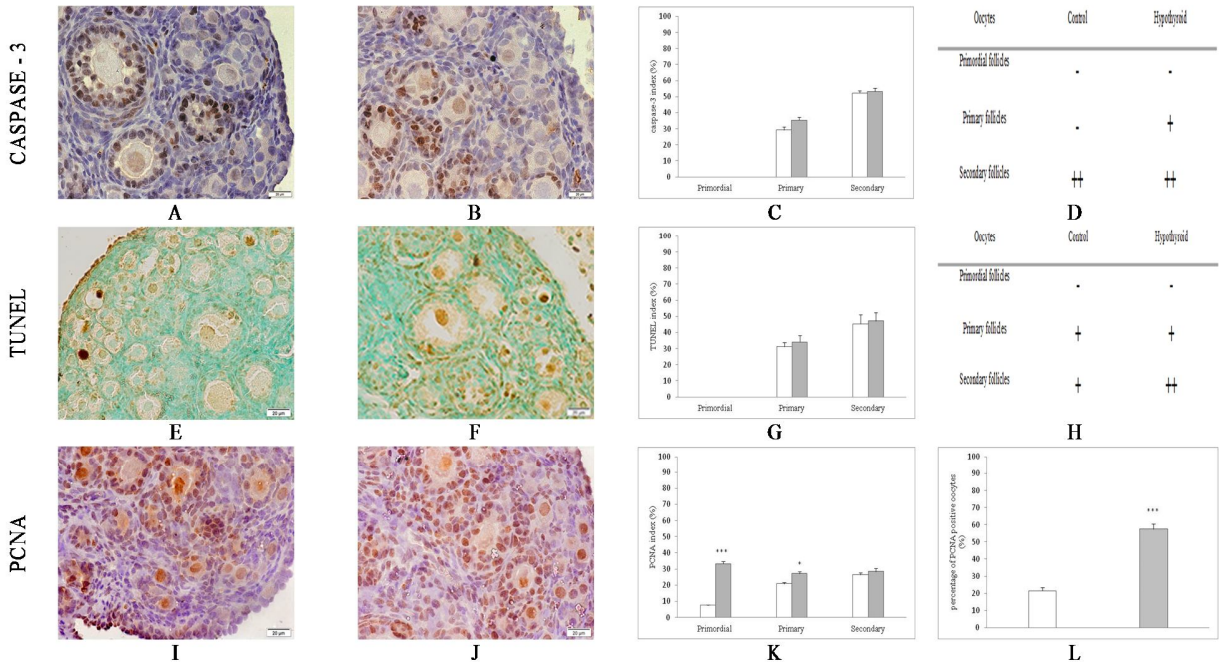

Figure 4. Caspase-3 expression in in ovaries of neonatal control (A) and hypothyroid rats (B). Caspase-3 index of granulosa cells in both groups (C). Semi quantitative assessment of caspase-3 positive oocytes in primordial, primary and secondary follicles of both groups (D). TUNEL expression in ovaries of neonatal control (E) and hypothyroid rats (F). TUNEL index of granulosa cells in both groups (G). Semi quantitative assessment of TUNEL positive oocytes in primordial, primary and secondary follicles of both groups (H). PCNA expression in ovaries of neonatal control (I) and hypothyroid rats (J). PCNA index of granulosa cells in both groups (K). Percentage of PCNA positive oocytes in primordial follicles of both groups (L). Open bars, control group; gray bars, hypothyroid group. Signal strength: (-) no signal, (+) weak, (++) moderate, (+++) severe. Chromogen diaminobenzidine (DAB), counterstain hematoxylin for caspase-3 and PCNA, methyl green for TUNEL. Bar 20µm. Results are presented as mean ± standard error (M ± SE). Levels of significance: *p<0,05; ***p<0.001.

Nucleolar and perinucleolar expression of caspase-3, TUNEL and PCNA was detected in ovaries of early infantile period in both groups of animals (Figure 5-A, B, E, F, I and J). In the early infantile period, granulosa cells in primordial follicles were caspase-3 negative in both the control and the hypothyroid group (Figure 5C). About one-third of granulosa cells in primary follicles and about one-half of secondary and preantral follicles were caspase-3 positive (Figure 5-C). Expression of cleaved caspase-3 was not detected in oocytes of primordial and primary follicles of both groups (Figure 5D). The expression of cleaved caspase-3 was weak in oocytes of secondary follicles of both groups as well as in oocytes of preantral follicles of control pups, while in the hypothyroid group, these oocytes were moderately stained (Figure 5-D). In the same period, about one-fifth of granulosa cells in primordial and primary follicles were TUNEL positive, while about one-half of them were positive in secondary and preantral follicles (Figure 5-G) in both groups of rats. TUNEL signal in oocytes was negative in primordial follicles, weak in primary follicles and moderate in secondary follicles (Figure 5-H). In preantral follicles, oocytes of the control group of rats expressed weak, while in oocytes in the hypothyroid group, a strong signal was detected (Figure 5-H). PCNA signal was not detected in granulosa cells of primordial follicles of both groups of animals (Figure 5-K). About one-third of granulosa cells in primary and about one-half in secondary and preantral follicles were PCNA positive in both groups of rats (Figure 5-K). A slightly higher number of PCNA positive oocytes was detected in the hypothyroid group of rats (Figure 5-L).

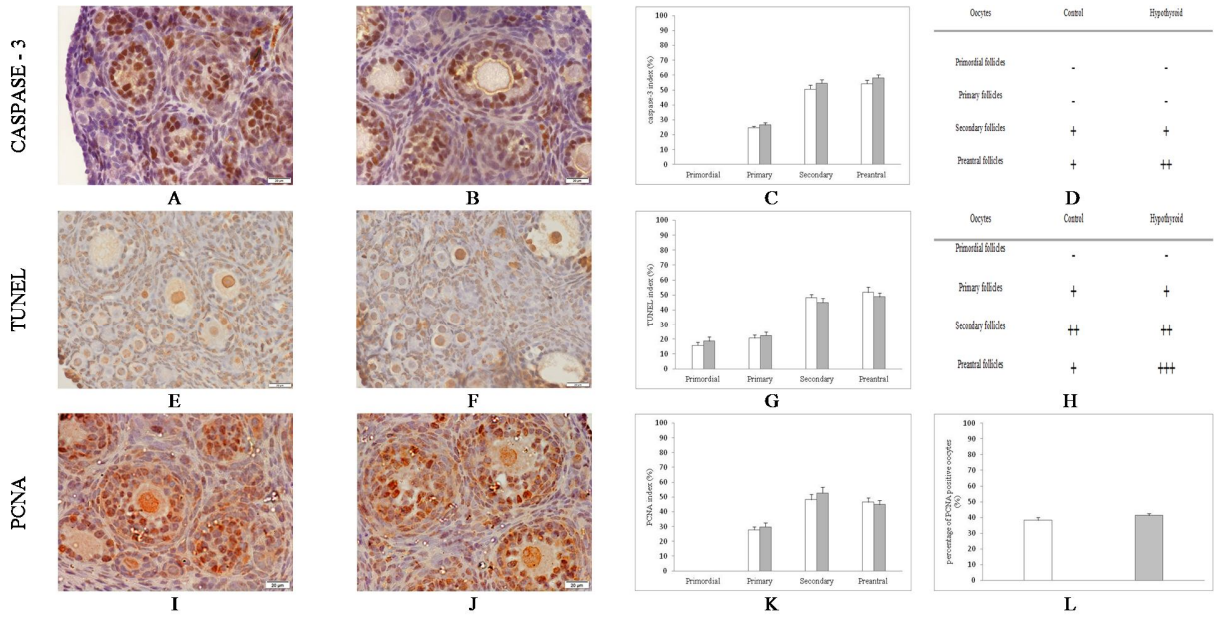

Figure 5. Caspase-3 expression in ovaries of early infantile control (A) and hypothyroid rats (B). Caspase-3 index of granulosa cells in both groups (C). Semi quantitative assessment of caspase-3 positive oocytes in primordial, primary, secondary and preantral follicles of both groups (D). TUNEL expression in ovaries of early infantile control (E) and hypothyroid rats (F). TUNEL index of granulosa cells in both groups (G). Semi quantitative assessment of TUNEL positive oocytes in primordial, primary, secondary and preantral follicles of both groups (H). PCNA expression in ovaries of early infantile control (I) and hypothyroid rats (J). PCNA index of granulosa cells in both groups (K). Percentage of PCNA positive oocytes in primordial, primary, secondary and preantral follicles of both groups (L). Open bars, control group; gray bars, hypothyroid group. Signal strength: (-) no signal, (+) weak, (++) moderate, (+++) severe. Chromogen diaminobenzidine (DAB), counterstain hematoxylin. Bar 20 µm. Results are presented as mean ± standard error (M ± SE).

4. Discussion

In this work, we have examined the effect of maternal hypothyroidism on early folliculogenesis, i.e. assembly and maintenance of primordial follicles in rat pups. These processes

take place in neonatal and early infantile period (between days four and seven) in rat pups. In humans, this process ends during prenatal development, in the third trimester of gestation (Fulton et al, 2005). To mimic the effect of human maternal hypothyroidism on early folliculogenesis, rat pups were maintained as hypothyroid in prenatal period and until the period of weaning i.e. infantile period. Hypothyroidism in all neonatal animals used in this work was confirmed by significantly increased thyroid gland activation index (Ia). A strong link between increased Ia and hypothyroidism was previously described (Rajab et al, 2015). Hypothyroid pups had a reduction of body mass by one-fourth compared to the controls, leading to the conclusion that decrease of TH during prenatal and neonatal period has a profound effect on overall cell proliferation and metabolism. The difference in body mass also became less important in early infantile period leading to the conclusion that induced hypothyroidism was mild and that tissues start to "find escape" from TH deficiency.

The main findings of this work are that during neonatal and early infantile period hypothyroid animals have: 1) premature formation of primordial follicles in developing ovarian cortex with dilated sER and altered mitochondrial morphology in oocytes; 2) a slightly higher number of apoptotic and three times higher number of PCNA positive oocytes and granulosa cells in primordial follicles and 3) slightly higher number of maturing primary, secondary, and preantral follicles without evident difference in apoptotic or cell proliferation markers. All of these findings indicate premature folliculogenesis with ultrastructural changes but relative/temporary resistance of all types of follicles to apoptosis in the neonatal and early infantile period due to maternal hypothyroidism.

All the main findings of this work are focused on primordial follicles. Entrance to meiotic profase I, transition from oogonia to primary oocyte, with a formation of squamous granulosa cells layer that surrounds oocytes all characterise follicle assembly and prepare them for long term quiescence, i.e., until selective activation in pre-pubertal and pubertal period (Eppig and Handel, 2011; Adhikari et Liu 2010). Our results have demonstrated that in hypothyroid rat pups this process is activated earlier than in control animals. This leads to the conclusion that decreased concentrations of TH induce premature proliferation of granulosa cells and differentiation of nests toward primordial follicles. Early infantile hypothyroid pups also have an increased number of primary and secondary follicles indicating that the whole process of folliculogenesis occurs faster, consequently inducing premature loss of follicles. In fact, the finding of premature folliculogenesis

is in accordance with our previous findings that 15, 30 and 60 days old hypothyroid rats have increased numbers of atretic follicles at all stages of development when compared to the controls (Radovanović, 1993; Radovanović, 2012). The phenomenon of premature activation of oocytes with complete elimination of all follicles in early maturity was also described in mice with specific deletion of *Pten* (Reddy et al., 2008) confirming that premature activation is detrimental to ovarian function in adulthood.

Our results also demonstrate that in neonatal and early infantile period oocytes and granulosa cells of primordial follicles have the impaired sER and mitochondrial morphology, but without nuclear condensation nor other signs of apoptosis on subcellular level indicating that these structural changes are not correlated with immediate cell death. Moreover, the percentage of apoptotic oocytes in primordial follicles is quite low and is only slightly different between hypothyroid and control pups in neonatal and early infantile period. Surprisingly, the level of PCNA expression was higher in oocytes and granulosa cells in primordial follicles of neonatal hypothyroid pups. The majority of primordial follicles in this period should stay inactive in an attempt to preserve oocytes until puberty. There is evidence that granulosa cells during the time of "relative quiescence" do not express PCNA (Picut et al., 2008) nor assimilate BrdU (Fenwick and Hurst, 2002). This is in accordance with our data from control animals and supports the view that quiescent state of oocytes is dependent on quiescent state of granulosa cells (McGee and Hsueh, 2000). It is known that PCNA is necessary for cellular DNA synthesis and cell cycle progression, as well as for DNA reparation (Jaskulski et al., 1988; Liu et al., 1989). We could suppose that PCNA expression indicates enhanced reparative processes in affected granulosa cells and oocytes in primordial follicles in neonatal hypothyroid pups.

In our study, dilatation of sER in neonatal hypothyroid rats could indicate stress response due to inadequate overall metabolic pathways as a result of TH deficiency, and as demonstrated in mitochondria, more fluid membranes due to altered fatty acyl composition (Brookes et al, 1998). Previously, it was also found that alterations in ER fatty acid and lipid composition result in the inhibition of sarco/endoplasmic reticulum calcium ATPase (SERCA) activity and ER stress that lead to its dilatation (Fu et al., 2011). Apart from the alteration in membranes, in order to manage the ER stress, unfolded protein response (UPR) increases ER lumen as a consequence of accumulation of its contents, particularly those that are synthesised as a stress-response (review: Schontal, 2012). Also, massive vacuolization, i.e dilatation of sER could precede autophagy-type

oocyte death in primordial follicles of adult rats (Escobar Sánchez et al., 2012). All these changes could lead to sER accumulation of Ca^{++} and more intense Alizarin red staining in neonatal ovaries of hypothyroid rats. Impaired sER function is also related to impaired mitochondria function and harmonised function of both organelles is important for normal intracellular Ca^{++} homeostasis (Báthori et al, 2006; Sano et al, 2009).

Although not explicitly shown in this study, altered mitochondrial morphology is connected to their impaired function (review: Galloway and Yoon, 2012). Impaired mitochondrial function sensitises cells to death via apoptosis (review: Shutt and McBride, 2012). Apparently, the consequence of impaired mitochondrial function is not visible in the neonatal and early infantile period of pups' development, but rather in late infantile, pre-pubertal, pubertal period, when apoptosis, i.e. atresia of follicles was detected (Radovanovic et al 2013). In neonatal and early infantile period, primordial follicles are resting and oocyte mitochondrial activity is lower comparing to later stages of folliculogenesis (Dumollard et al., 2006). In fact, it is also known that altered mitochondrial morphology precedes activation of caspase-3 (review: Galloway and Yoon, 2012). We could suppose, then, that despite alteration in sER and mitochondria, oocytes could be protected from apoptosis until a rise in LH and FSH would stimulate glucose consumption and anabolic state of the follicles (review: Sutton-McDowall et al., 2010). This anabolic state, in the case of hypothyroidism and altered sER and mitochondria, would lead oocytes and granulosa cells into inevitable apoptosis and atresia (Radovanovic et al., 2012). The altered mitochondrial morphology was also found in neonatal, infantile, and pubertal cerebellar neurons of rats made hypothyroid due to maternal hypothyroidism (Singh et al., 2003). Their altered morphology induced release of apoptogenic proteins and was connected to extensive apoptosis during postnatal neurogenesis (Singh et al., 2003). At this point, we can conclude that in the rat model that mimics human maternal hypothyroidism, mechanisms of damage to tissues/cells that have no possibility to renew in postnatal development converge to alteration of sER and mitochondria, possible Ca^{++} misbalance, and subsequent proneness to apoptosis.

Our results also demonstrate that in all experimental conditions apoptosis is visible only in primary and secondary follicles that are predestined to disappear in a first wave of folliculogenesis, indicating that this process is not affected in hypothyroidism.

5. Conclusion

Considering all of the findings that our group has gathered concerning the effect of mild maternal hypothyroidism on the development of offspring's female gonads, we can hypothesise that alterations on sER and mitochondrial level that exist in the pool of prematurely formed resting primordial follicles could be without clinical signs during infancy, but could represent an underlying cause of potential female reproductive problems in sexually mature age.

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