

# Effect of Hypothyroidism on Growth, Viability, and Characteristics of Ascitic Ehrlich Tumor Cells in Ovariectomized and Non-ovariectomized Mice

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## ABSTRACT

The effect of hypothyroidism on the ascitic form of Ehrlich tumor in either non-ovariectomized or ovariectomized adult female mice was studied.

Hypothyroidism was induced by treatment with propylthiouracil (PTU). Forty mice were divided into four groups: ovariectomized hypothyroid, non-ovariectomized hypothyroid, ovariectomized euthyroid, and non-ovariectomized euthyroid. Mice were inoculated intraperitoneally with a suspension of  $29 \times 10^6$  cells and tumor

growth curve was determined by measuring abdominal circumference for 10 days. At the end of the experimental period, mice were sacrificed. Hypothyroidism resulted in lower abdominal circumference and lower volume of ascitic liquid. However, hypothyroidism did not affect the viability or concentration of tumor cells, the nucleus-cytoplasm ratio, or the mean nuclear diameter of neoplastic cells. The number of nucleolar organizer regions (NOR) of neoplastic cells did not differ among groups.

In conclusion, hypothyroidism, regardless of the functional state of the gonads, resulted in a delayed growth of the ascitic form of Ehrlich tumor as it reduced the liquid volume and did not reduce the number, viability, or diameter of neoplastic cells.

**Table 1.** Abdominal Circumference (cm) (mean  $\pm$  SD) in Hypothyroid and Euthyroid Female Mice, Ovariectomized and Non-ovariectomized, with Ascitic Ehrlich Tumor on Days 0, 2, 4, 6, 8, and 10 after Inoculation

Measurement day	Hypothyroid		Euthyroid	
	Ovariectomized (n = 10)	Non-ovariectomized (n = 10)	Ovariectomized (n = 10)	Non-ovariectomized (n = 10)
Day 0 (before inoculation)	9.47 $\pm$ 0.54	9.17 $\pm$ 0.81	9.29 $\pm$ 0.50	9.34 $\pm$ 0.89
Day 2 after inoculation	9.39 $\pm$ 0.52 a	8.90 $\pm$ 0.70 b	9.5 $\pm$ 0.46 a	9.68 $\pm$ 0.76 a
Day 4 after inoculation	9.57 $\pm$ 0.58 b	8.88 $\pm$ 0.90 c	10.19 $\pm$ 0.60 a	10.31 $\pm$ 0.46 a
Day 6 after inoculation	10.37 $\pm$ 0.71 a	9.67 $\pm$ 0.54 b	10.85 $\pm$ 0.60 a	10.51 $\pm$ 0.56 a
Day 8 after inoculation	10.02 $\pm$ 0.72 b	9.61 $\pm$ 0.68 b	11.39 $\pm$ 0.53 a	11.03 $\pm$ 0.42 a
Day 10 after inoculation	10.25 $\pm$ 0.40 b	9.77 $\pm$ 0.88 b	11.60 $\pm$ 0.73 a	11.28 $\pm$ 0.61 a

Different letters in the same row indicate a statistically significant difference (P<0.05).

## INTRODUCTION

Breast cancer is the most common neoplasia in women.<sup>1,2</sup> Its etiology is multifactorial and involves the participation of genetic, environmental, infectious, nutritional, and, mainly, hormonal factors.<sup>1,3</sup>

The first report associating thyroid dysfunction with breast cancer dates from 1896, when the Scottish physician Dr George Beatson treated breast cancer with ovariectomy and administration of thyroid extract.<sup>4</sup> Since then, several studies have shown a correlation between an increased breast cancer incidence and hypothyroidism,<sup>5</sup> hyperthyroidism,<sup>6</sup> and even with hormone replacement with thyroxine (T4).<sup>7</sup>

Hypothyroidism is a common endocrine disorder in individuals of all ages<sup>8</sup> and it is the most common thyroid dysfunction in individuals with breast cancer.<sup>9,10</sup> Some studies have suggested that the lack of thyroid hormones delays neoplastic growth and improves chemotherapeutic treatment prognosis.<sup>11</sup> Other studies have suggested that thy-

roid hormones do not influence the development of breast cancer since women with thyroid dysfunction do not present with altered tumor development.<sup>12,13</sup> Most studies of this subject have been epidemiologic investigations, which may explain the discrepancies among the results. Hypothyroidism in women is often associated with nonfunctioning gonads.<sup>14,15</sup> Lack of sex hormones decreases the risk of breast cancer in women.<sup>16</sup> Thus, the study of a possible interrelationship between sex hormones and thyroid hormones in breast cancer is pertinent.

Ehrlich tumor is a species-specific, transplantable neoplasia from malignant epithelium that corresponds to mice's mammary adenocarcinoma. It grows in several strains of this animal species: in an ascitic form when inoculated in the peritoneal cavity and in the solid form when subcutaneously inoculated.<sup>17</sup> In order to elucidate factors involved in carcinogenesis, several researchers have been using transplantable experimental tumors to study the effect of chemicals,



**Figure 1.** Female mice, ascitic Ehrlich tumor. From left to right: (A) non-ovariectomized euthyroid and non-ovariectomized hypothyroid, and (B) ovariectomized euthyroid and ovariectomized hypothyroid. Abdominal volume decreased in hypothyroid groups compared with euthyroid animals.

viruses, and hormones on carcinogenesis. The use of transplantable neoplasias provides researchers with previous knowledge of the amount of tumor cells inoculated and their early characteristics. In addition, rapid neoplasia development decreases study time.

The main purpose of this study was to induce hypothyroidism in ovariectomized or non-ovariectomized adult female mice in order to create an experimental model that allowed the study of the effect of hypothyroidism on cell growth, viability, and characteristics of ascitic Ehrlich tumor from mammary adenocarcinoma.

## MATERIALS AND METHODS

Forty 3-month-old female Swiss strain mice were used. Animals were kept in plastic cages (10 animals/cage) and received commercial diet and water ad libitum. The animals were submitted to 12 hours of light and 12 hours with no light, and were distributed in 4 groups of 10 animals. Two groups were submitted to bilateral ovariectomy and 2 were not ovariectomized.

Hypothyroidism was induced 10 days after ovariectomy in 1 group of ovariectomized animals and 1 group of non-ovariectomized animals to create 4 treatment groups: (1) ovariectomized hypothyroid, (2) non-ovariectomized hypothyroid, (3) ovariectomized euthyroid, and (4) non-ovariectomized euthyroid (control). Animals were induced to hypothyroidism by daily administration of propylthiouracil (PTU; Sigma Aldrich, St. Louis, MO) in the animals' drinking water (1mg/mL). Hypothyroidism induction began 30 days before the inoculation of tumor cells and was maintained until the end of the experiment. Animals from the euthyroid group received distilled water as placebo. The amount of PTU ingested daily was estimated for each animal; the daily amount of water consumed was inferred from the amount of water provided, minus the value found divided by the number of animals in the cage.

Ascitic Ehrlich tumor cells were used in Swiss strain mice. The cells were maintained in laboratory of Department of Pathology of Institute of Biological Sciences of Federal University of Minas Gerais, Brazil. Mice from all groups received an intraperitoneal injection of cell suspension containing  $29 \times 10^6$  Ehrlich tumor cells. In order to evaluate the tumor growth curve, abdominal circumference was measured with a measuring tape at baseline and every 2 days after tumor inoculation, for a total of 6 measurements.

**Table 2.** Measured Variables in Ascitic Ehrlich Tumor (mean  $\pm$  SD) in Female Mice, Hypothyroid and Euthyroid, Ovariectomized and Non-ovariectomized, 10 Days after Tumor Inoculation

Variable	Hypothyroid		Euthyroid	
	Non-Ovariectomized (n = 10)	Non-ovariectomized (n = 10)	Ovariectomized (n = 10)	ovariectomized (n = 10)
Ascitic liquid volume (mL)	5.00 $\pm$ 0.74 b	3.50 $\pm$ 1.96 b	8.22 $\pm$ 2.29 a	8.75 $\pm$ 2.55 a
No. of tumor cells/mL	159.55 $\pm$ 20.13	153.25 $\pm$ 25.88	131.60 $\pm$ 32.50	124.90 $\pm$ 35.70
Percentage of viable tumor cells/mL (%)	96.98 $\pm$ 1.54	97.61 $\pm$ 1.36	95.50 $\pm$ 3.46	94.90 $\pm$ 2.30
Ascitic liquid pH	7.20 $\pm$ 0.26	7.15 $\pm$ 0.24	7.00 $\pm$ 0.33	7.10 $\pm$ 0.21
Ascitic liquid total proteins (mg/dL)	5.54 $\pm$ 0.74	5.32 $\pm$ 1.17	4.94 $\pm$ 1.01	5.25 $\pm$ 0.86

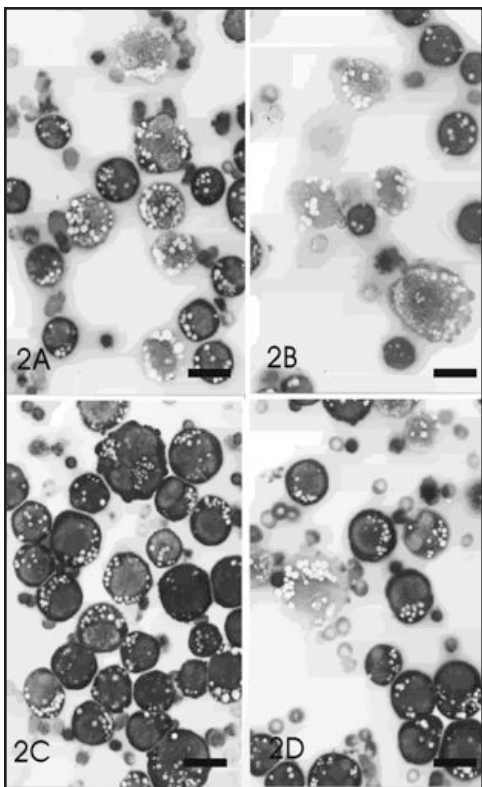
Different letters in the same row indicate a statistically significant difference (P<0.05).

After 10 days of tumor implantation, animals were necropsied in order to inspect and collect abdominal and thoracic organs and lumbar and mesenteric lymph node to study metastasis. Thyroid morphology was carried out in order to verify the effect of PTU on the gland. Organs collected at necropsy were fixed in formaldehyde 10% and processed using the paraffin inclusion technique. Histologic sections were stained with hematoxylin-eosin. Ascitic liquid volume was measured. Ascitic liquid was also collected in order to determine pH, quantify total proteins, and count and to study the viability of tumor cells according to the following procedure.

Liquid was centrifuged (3,000 rpm) for 3 minutes, supernatant was discharged, and cells were resuspended in physiological saline. This procedure was repeated 3 times in order to obtain a clear liquid, correspondent to a cell suspension with a minimum of fibrin and red blood cells. Then tumor cells were counted and a test of their viability was carried out as follows: 20  $\mu$ L of cell suspension were withdrawn and this frac-

tion was added to a tube containing 1,980  $\mu$ L physiological saline. After homogenizing it, 100  $\mu$ L of this cell suspension was added to 100  $\mu$ L of Trypan Blue stain at 0.1%. Cells were counted using a Neubauer hemocytometer in 4 external quadrants. Cells stained by Trypan blue were considered inviable and translucent cells were considered viable. The formula used to determine number of viable cells was deduced previously described<sup>21</sup> [no. of cells/mL = no. of counted cells  $\times$  correction factor]. [Correction factor = (chamber depth factor  $\times$  dilution factor)/counted area], where depth factor = 10, correction factor =  $5 \times 10^5$ , dilution factor = 200, counted area = 4 mm<sup>2</sup>, and conversion from mm<sup>2</sup> to mL =  $\times 10^3$ ].

Smears of neoplastic cell suspension from each animal were stained with Panotico stain (Laborclin Products LTDA, Pinhais, Paraná, Brazil) and Giemsa. The ratio of clear cells:dark cells and nucleus:cytoplasm, and the mean diameter of neoplastic cell nuclei were determined. Smears were also impregnated with silver in order to individualize nucleolar organizer regions



**Figure 2.** Smears of ascitic liquid cells of ascitic Ehrlich tumor in female mice. Panotico stain, Magnification 1071 $\times$ . (A) Non-ovariectomized and (B) ovariectomized euthyroid groups. Clear cells, round, with abundant cytoplasm, eosinophilic and very vacuolated, with little defined edges with little defined edges and distinct and lobulated nuclei and, in a smaller number, dark cells with moderate cytoplasm, basophilic, with few vacuoles, with defined edges and hyperchromatic nuclei. (C) Non-ovariectomized and (D) ovariectomized hypothyroid groups. Prevalence of dark round cells, with moderate cytoplasm, basophilic, with few vacuoles, with defined edges and hyperchromatic nuclei.

(NORs), in accordance with a technique previously described<sup>18</sup> with some modifications proposed by Aubele et al.<sup>19</sup>

With an ocular containing a ruler, mean diameter (longitudinal and transversal) of 30 randomly chosen cells and their nuclei were measured in immersion objective. In the end, a correction

factor, obtained by a micrometric slide scale, was applied to means.

An immersion objective was used to count NORs present in 30 nuclei of randomly chosen neoplastic cells. Only those NORs that were individualized with black or brown dots were counted. When they were clustered, they were considered as a single NOR.<sup>20</sup> Data were submitted to analysis of variance (ANOVA) from the Statistical Analysis System (Cary, NC) and means were compared by Student-Newman-Keuls test (SNK).

## RESULTS

According to estimated PTU consumption, ovariectomized and non-ovariectomized hypothyroid animals consumed about 2.48 mg and 2.63 mg of PTU/day, respectively, with no statistically significant difference between groups. Thyroid morphology of hypothyroid animals, regardless of gonad functional status, was characterized by the presence of parenchymatous goiter with intra- and interfollicular adenomatosis. Moreover, animals were apathic and had dry, ruffled, and small amount of hair, differing from the group not treated with PTU.

In the beginning of the experiment, before the inoculation of tumor cells, there was no significant difference in the abdominal circumference of animals in any groups (Table 1). From the second day on, however, the effect of hypothyroidism was verified in the retardation of tumor growth that it was initially significant only in the non-ovariectomized hypothyroid group. From the fourth day on, tumor growth in ovariectomized and non-ovariectomized hypothyroid groups was significantly lower until the end of the experiment (the tenth day) (Table 1 and Figure 1).

Hypothyroid animals presented significantly decreased ascitic liquid volume. Interestingly, despite this decrease, hypothyroidism did not change the via-

**Table 3.** Nucleolar Organizer Regions (NORs) Number, Ratio Dark:Clear Cells, Mean Nuclear Diameter ( $\mu\text{m}$ ) and Ratio of Nucleus:Cytoplasm (mean  $\pm$  SD) in Smears of Ascitic Ehrlich Tumor in Female Mice, Hypothyroid and Euthyroid Ovariectomized and Non-ovariectomized, 10 Days after Tumor Inoculation

Variable	Hypothyroid		Euthyroid	
	Ovariectomized (n = 10)	Non-ovariectomized (n = 10)	Ovariectomized (n = 10)	Non-ovariectomized (n = 10)
No. of NORs/nucleus	17.43 $\pm$ 2.48 b	21.34 $\pm$ 2.29 a	19.22 $\pm$ 2.47ab	19.92 $\pm$ 3.53ab
No. of dark cells/field	58.80 $\pm$ 20.76 a	62.48 $\pm$ 22.51 a	38.20 $\pm$ 17.33 b	37.45 $\pm$ 13.08 b
No. of clear cells/field	4.45 $\pm$ 1.16	3.65 $\pm$ 2.21	4.12 $\pm$ 1.30	3.63 $\pm$ 1.12
Ratio of dark:clear cells/field	13.48 $\pm$ 4.65 b	21.00 $\pm$ 8.28 a	9.42 $\pm$ 3.80 b	11.12 $\pm$ 4.,66 b
Mean nuclear diameter ( $\mu\text{m}$ )	12.41 $\pm$ 0.93	12.23 $\pm$ 0.58	11.97 $\pm$ 0.79	11.63 $\pm$ 0.76
Ratio of nucleus:cytoplasm	3.57 $\pm$ 0.30	3.48 $\pm$ 0.41	3.81 $\pm$ 0.41	3.37 $\pm$ 0.45

Different letters in the same row indicate a statistically significant difference ( $P < 0.05$ ).

bility and number of tumor cells/mL, or the amount of total proteins and ascitic liquid pH. Ovariectomy did not significantly change the ascitic liquid volume (Table 2).

In the analysis of tumor cell suspension smears in euthyroid groups, most cells were clear, round, with abundant cytoplasm, eosinophilic and very vacuolated, with poorly defined edges; they had distinct and lobulated nuclei and, in a smaller number, dark cells (Figure 2A and B). Hypothyroid groups presented a larger amount of dark round cells, with moderate cytoplasm, basophilic, with few vacuoles, with defined edges and hyperchromatic nuclei, most of them oval (Figure 2C and D). Morphometric analysis confirmed that the ratio of dark:clear cells was significantly higher in the non-ovariectomized hypothyroid group (Table 3).

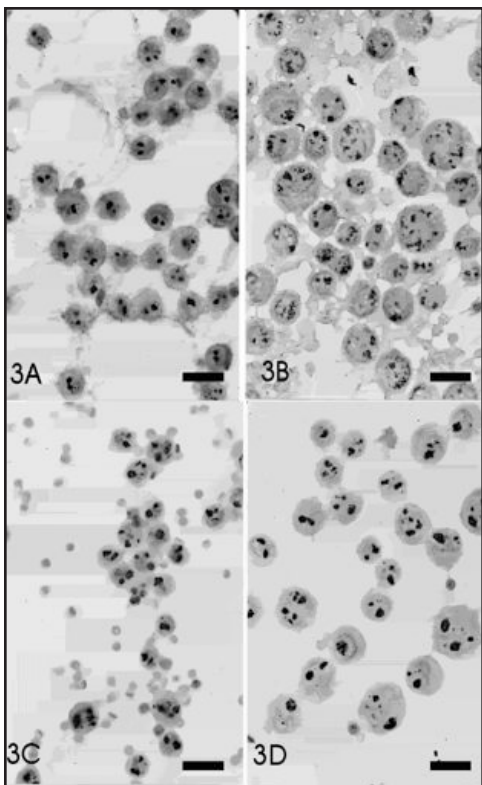
No significant difference was observed in the mean nuclear diameter and in the nucleus:cytoplasm ratio

between groups. Regardless of the group, NORs were strongly connected, constituting large argiophilic structures, round or irregular, thoroughly filling the nucleolus, and in some cases, small NORs could be seen dispersed in the nucleus. No significant difference was observed in the number of NORs in neoplastic cells from hypothyroid groups when compared with those from euthyroid groups (Table 3 and Figure 3).

In all groups, no metastases were observed in spleen, liver, kidneys, heart, and lungs. There was only a large amount of neoplastic cells on the capsule of these organs. Only 1 non-ovariectomized hypothyroid animal and 2 ovariectomized hypothyroid animals presented metastases in the mesenteric lymph node and in the lumbar lymph node, respectively.

## DISCUSSION

Thyroid morphology of animals treated with PTU was characterized by the pres-



**Figure 3.** Smears of ascitic liquid cells of ascitic Ehrlich tumor in female mice. Impregnation of argiophilic nucleolar organizer regions (NORs). Magnification 1071x. (A) Non-ovariectomized and (C) ovariectomized euthyroid groups, and (D) ovariectomized hypothyroid group. NORs strongly connected, constituting large argiophilic structures, round or irregular, thoroughly filling the nucleolus, and in some cases, small NORs dispersed in the nucleus. (B) Non-ovariectomized hypothyroid group with small NORs and in larger numbers.

ence of parenchymatous goiter with intra- and interfollicular adenomatosis. These findings corroborate the hypothyroid-inducing action of PTU. Furthermore, clinical signs of changes in fur and behavior corroborate the success of hypothyroidism induction with PTU. Several studies were able to induce hypothyroidism successfully using the same dose and the same method used to administer PTU in this study.

Hypothyroid animals had a significant decrease in ascitic liquid volume.

Interestingly, despite this decrease, hypothyroidism has not changed viability and number of tumor cells/mL, as well as the amount of total proteins and ascitic liquid pH. Ascitic liquid production in Ehrlich tumor has been credited mainly to hyperpermeability and vessels from peritoneal cavity microcirculation.<sup>22</sup> This vascular change is due to the action of neoplastic cells, which induce an increased expression of receptors to autocrine motility factor (AMF). At the same time, they secrete a large amount of this factor. The link between AMF and its receptor induces angiogenesis and changes vascular permeability as it changes the morphology of endothelial cells, with a subsequent formation of a large amount of ascitic liquid.<sup>23</sup> Based only on the results found, however, it is not possible to conclude whether there are changes of these mechanisms occurring in hypothyroidism, culminating in a lower production of ascitic liquid.

In contrast with euthyroid groups, hypothyroid groups presented tumors with a larger amount of dark cells, and the noncastrated hypothyroid group presented ratios of dark cells:clear cells that were significantly higher than smears of ascitic liquid. Although there are no classifications of Ehrlich tumor cells as dark and clear in the literature, this seem to be the most appropriate denomination considering the morphological characteristics observed. Cytoplasm vacuoles from clear cells suggest some type of secretory activity, but nothing is mentioned in the literature about such a hypothesis. Negative significant correlation between the number of dark cells and volume of ascitic liquid reinforces the suspicion that clear cells may be involved in the production of ascitic liquid. But this hypothesis needs further investigation.

There was no significant difference among groups regarding the number of NORs in neoplastic cells. The quantifica-

tion of NORs, chromosomal DNA loops where ribosome genes are coded, has been widely employed as indicators of cell proliferation. NORs present diagnostic and prognostic value in tumor pathology.<sup>20,24</sup> The number of NORs in the nucleus of malignant neoplastic cells has been shown to be significantly higher compared with the number of NORs in normal, reactive, or neoplastic benign cells.<sup>25</sup> It is intriguing that in the hypothyroid groups, which presented a lower level of tumor growth, the number of NORs in tumor cells did not differ significantly among the other groups. Characteristics of neoplastic cells in hypothyroid groups, with regard to cell number and viability, nucleus diameter, nucleus:cytoplasm ratio, and the number of NORs, suggests that hypothyroidism decreased tumor size by reducing ascitic liquid volume and not by reducing the malignancy of cells. This result is in contrast with the hypothesis formulated in the beginning of this study, that hypothyroidism would decrease Ehrlich tumor growth by reducing tumor cells malignancy. In the solid form of this tumor, hypothyroidism has also decreased tumor growth, without reducing cells' grade of malignancy.<sup>26</sup> Studies about cell apoptosis rate are under way in solid tumors to verify whether hypothyroidism reduces Ehrlich tumor growth by increasing the apoptosis rate (data not shown).

Although Ehrlich tumor cells have been shown to be responsive to sex hormones,<sup>27,28</sup> we didn't observe any influence of ovariectomy upon ascitic Ehrlich tumor cells.

In conclusion, hypothyroidism, associated or not associated with ovariectomy, delays ascitic Ehrlich tumor growth, because it decreases its liquid volume and not because it decreases viability, nucleus:cytoplasm ratio, and number of NORs from neoplastic cells. We may also conclude that in hypothyroidism,

ascitic Ehrlich tumor presents a larger prevalence of dark and slightly vacuolated cells and that ovariectomy does not change tumor growth and its cell characteristics.

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