

**Effect of Maternal Thyroid Activity Upon
In Vitro Protein Synthesis in the Rat Blastocyst**

VERNON G. ARCHER, Department of Biology
Jackson State University, Jackson, Mississippi 39203

and

JAMES P. HOLLAND, Department of Biology
Indiana University, Bloomington, Indiana 47405

Introduction

Many investigators have reported that thyroid hormones influence the reproductive system and pregnancy. The literature contains conflicting reports concerning the extent and effect of this influence. Examples of these conflicts and possible explanations were presented in an earlier publication (10) and have been reviewed by Leatham (13). Despite numerous investigations into this phenomenon, the mechanism by means of which thyroid hormone exerts its effect upon reproduction and pregnancy remains unresolved.

In our laboratory, earlier studies of this phenomenon utilized the technique of experimentally delayed implantation of blastocysts (6) which allows the investigator to control some of the variables in studies of reproduction. Using this technique, it was demonstrated (10 and 12) that in rats receiving daily injections of either 48 μg or 8 μg L-thyroxine, the hyperthyroid condition was able to significantly counteract the detrimental effects of progesterone deficiency upon the number of surviving blastocysts on Day 8 of pregnancy or the number of implanting blastocysts on Day 13 of pregnancy. Surgical thyroidectomy had the opposite effect. The thyroid effect was shown to be exerted during the progesterone-dependent delay period rather than during the estrogen-dependent implantation process. Also, our earlier work (11) showed that the sub-normal levels of uterine alkaline phosphatase which occur during progesterone deficiency were restored to normal by thyroxine treatment, but were further depleted by a hypothyroid condition in rats examined at a time corresponding to Day 8 of pregnancy (the final day of the progesterone-dependent delay period). It was further demonstrated that the thyroxine effect on uterine alkaline phosphatase was not due to a generalized action on metabolism. Thus, thyroid hormone does exert an influence upon blastocyst survival by means of a modification of progesterone-dependent uterine physiology, but the possibility also exists that thyroid hormone may exert a more direct influence upon the blastocyst itself. The present investigation was designed to study this possibility by examining protein and nucleic acid metabolism of blastocysts during alterations of the progesterone and thyroid hormone levels.

Materials and Methods

Sprague-Dawley-derived female albino rats, between 60 and 100 days old were maintained on Purina Laboratory Chow and tap water ad libitum. Rats were housed in an animal room at 24°C with a daily illumination schedule of 14 hours of light and 10 hours of darkness. Females showing a proestrus or early oestrus vaginal smear were placed overnight in cages with adult male rats. Insemination was confirmed on the following morning by the presence of spermatozoa in the vaginal smear and this was designated as Day 1 of pregnancy. Hyperthyroidism was induced by daily injection of 12 μg L-thyroxine beginning at least ten days

prior to insemination. Surgical thyroidectomies were performed through a midventral incision in the neck at least four weeks prior to insemination.

Blastocyst Collection and Incubation

Blastocysts were collected from control, hyperthyroid and hypothyroid animals which were sacrificed on Day 5 of pregnancy. The uterus was excised, the cornua were severed from the vagina, and each horn was flushed using a 23 gauge hypodermic needled fitted to a one-ml syringe containing Basic Medium for Ova Culture-2 (2). The blastocysts were flushed into depression slides using a minimal volume of BMOC-2. Finely drawn Pasteur pipettes were then used to place the blastocysts in culture dishes for incubation in BMOC-2 containing the radioactive precursors. The BMOC-2 was previously sterilized by filtration through a millipore filter.

Approximately 0.5 ml of culture medium was placed in a 60 x 15 style 3010 plastic organ tissue culture dish (Falcon Plastics, Oxnard, Calif.), equipped with an absorbent ring which was saturated with water to provide the inside of the dish with water vapor. Uniformly labeled ^{14}C -leucine (specific activity between 1 and 3mCi/mM) was added to the BMOC-2 to give a concentration of 1×10^5 mM. Between 10 and 20 blastocysts were placed in each dish and the dishes were placed for 1.5 hours in a Model 3212 water-jacketed incubator (National Incubator) at 37°C with a $\text{CO}_2\text{-O}_2$ atmosphere. To verify protein synthesis, incubations were done in both the presence and absence of cycloheximide (5 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$).

^{14}C -Amino Acid Uptake and Incorporation

At the end of the incubation period, the blastocysts were collected in approximately 5 μl of medium and rapidly washed by means of sequential transfers through three changes of two ml of BMOC-2 containing non-radioactive leucine. The blastocysts were then transferred in approximately 5 μl of the final wash to a 2 ml centrifuge tube containing 100 μl of water to cause cellular lysis. Using dry ice and acetone, the water containing the blastocysts was frozen and thawed three times, and was agitated on a vortex mixer during each thawing. One mg of bovine serum albumin and 1 mg of glycogen in 20 μl of water were added to each tube to act as cold carrier and thus reduce the loss of labeled compounds during the experimental manipulations (3).

The protein from the blastocysts was precipitated, extracted and prepared for counting according to the method reported by Brinster (3) with the exception that Aquasol was used as the scintillation fluid. This procedure involved precipitation of the protein with 5% trichloroacetic acid (TCA) and counting the TCA-washed precipitate (acid insoluble) as the amount of the ^{14}C -leucine which was incorporated into protein. An aliquot of the TCA supernatant (acid soluble) which had been drawn off and measured also was counted and used to calculate the radioactivity of the entire supernatant or acid soluble fraction. The sum of the ^{14}C -radioactivity in the acid insoluble (incorporated) and acid soluble fractions was considered to be the total ^{14}C uptake (total uptake = incorporated + acid soluble). All counts of radio-activity were made using a Model LS-233 liquid scintillation counter.

Autoradiography

Delayed blastocysts were obtained as described by Holland et al. (12) involving ovariectomy on Day 3, daily injection of progesterone (2.0 mg/day or 0.4 mg/day), and flushing of the uteri on Day 8 of pregnancy. The blastocysts were in-

cubated using the same procedure as described above with the exception that the radioactive precursors which were added to the BMOC-2 (at a concentration of 1×10^{-4} mM) were ^3H -uridine (6 uCi/ml, 20-25 (Ci/mole) or ^3H -phenylalanine (6 uCi/ml, 170-200 mCi/mM) for a five hour incubation period. Studies were also done using a concentration of $1 \mu\text{M}$ of ^3H -uridine in the incubation medium since this has been shown (7) to be a saturation level which eliminates differences in incorporation which may be associated with the concentration of precursor in the medium. Some incubations were performed in the presence of actinomycin D ($0.1 \mu\text{g/ml}$) or cycloheximide ($5 \mu\text{g/ml}$ or $50 \mu\text{g/ml}$). After incubation the blastocysts were removed and washed as described earlier. Several blastocysts from each treatment group were placed on separate microscope slides which had been subdued with albumen and were prepared for autoradiography in a manner similar to that described by Skalko (16) using Carnoy's fixative. Blastocysts were treated with 2% perchloric acid at 4°C for 20 minutes to remove free nucleotides and some slides were subjected to ribonuclease extraction (1 mg/ml at 37°C for 2 hours) to confirm the specificity of incorporation of the uridine. Slides were coated with a 1:1 dilution of Kodak Nuclear Emulsion (NTB-3, exposed for seven days), developed in Kodak D-19 for four minutes at 18°C , acid fixed, and stained with hematoxylin and eosin.

Results

Table 1 summarizes the data from *in vitro* studies concerning ^{14}C -leucine uptake and incorporation into protein by blastocysts which had been flushed from uteri of thyroidally altered rats on Day 5 of pregnancy. When compared with blastocysts from control (euthyroid) rats, the blastocysts from both hyperthyroid and from hypothyroid rats showed a significantly greater uptake and incorporation of ^{14}C -leucine. However, the percent of the ^{14}C -leucine uptake which had been incorporated into protein by these blastocysts was approximately twice the control level in blastocysts from hyperthyroid rats and was only about one-half the control level in blastocysts from hypothyroid rats. These differences were statistically significant. Since there was low incorporation of the amino acid in the blastocysts from hypothyroid animals, the disproportionally high uptake is possibly associated with extracellular movement of fluids which occurs during hypothyroidism. In this case, possibly there is movement of fluid (containing radioactive leucine) into the cavity of the blastocyst and thus a disproportionally high uptake is registered for these blastocysts.

TABLE 1. *In Vitro Uptake and Incorporation of ^{14}C Leucine by Blastocysts From Thyroidally Altered Rats*

| Thyroid State | Total Uptake (cpm/blastocyst) | Incorporation (cpm/blastocyst) | %Incorporation of Uptake into Protein |
|---------------|----------------------------------|-----------------------------------|--|
| Euthyroid | 24.40 \pm 3.50 | 6.42 \pm 0.28 | 26.3 \pm 4.1 |
| Hyperthyroid | 43.12 \pm 7.50* | 27.62 \pm 3.78* | 64.1 \pm 8.3* |
| Hypothyroid | 103.64 \pm 8.95* | 13.90 \pm 2.88* | 13.4 \pm 2.2* |

*Significant at the 5% level by "t" test

(Each thyroid state represents results from five groups consisting of 15-20 blastocysts/group.)

Table 2 summarizes the data from the autoradiographs of experimentally delayed blastocysts which had been incubated for five hours in BMOC-2 containing ^3H -phenylalanine or ^3H -uridine. Blastocysts from euthyroid rats showed essentially identical amounts and distribution of the precursors regardless of whether these rats were receiving a low or normal maintenance dose of progesterone. Blastocysts from hyperthyroid rats clearly showed increased amounts of both precursors in the inner cell mass (embryonic disc). The changes in the trophoblast area were less clearly defined but tended to show a slightly decreased concentration. Blastocysts from thyroidectomized rats of both progesterone treatment levels generally showed decreased incorporation of precursors in the trophoblast but those blastocysts from the thyroidectomized rats which had been maintained on a deficiency dosage of progesterone (0.4 mg/day) showed very low levels of both precursors in the trophoblast and showed an absence of phenylalanine in the inner cell mass. A striking observation is the fact that these were the only blastocysts in the entire experiment which showed a total or complete absence of uridine in the inner cell mass. The same pattern of uridine distribution was observed with both concentrations of uridine tested. Cyclohexamide (5 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$) caused a 40% and 100% inhibition, respectively, of leucine incorporation. Actinomycin D (0.01 $\mu\text{g}/\text{ml}$ and 0.1 $\mu\text{g}/\text{ml}$) caused 44% and 88% inhibition, respectively, of ^3H -uridine incorporation and a marked depression of ^3H -phenylalanine into protein after three hours of incubation.

Discussion

Earlier studies (12) in our laboratory demonstrated that thyroxine in dosages as low as 8 $\mu\text{g}/\text{day}$ was able to counteract the detrimental effects of progesterone deficiency upon survival of blastocysts during experimentally induced delay of implantation. On the other hand, thyroidectomy worsened the effects of progesterone deficiency. Although it is known that the diapausing blastocyst shows decreased metabolic and synthetic activities (15) our earlier work demonstrated that the thyroid effect upon the blastocyst is exerted during the progesterone-

TABLE 2. *Summary of Autoradiographs Showing In Vitro "Incorporation" of Radioactive Precursors into Experimentally Delayed Blastocysts from Progesterone-Maintained Rats with Altered Thyroid Activity*

| Experimental Group | Relative Concentration of Precursor | | | |
|----------------------------------|-------------------------------------|---------|-----------------|---------|
| | Trophoblast | | Inner Cell Mass | |
| | Phenylalanine | Uridine | Phenylalanine | Uridine |
| Euthyroid 2.0 mg progesterone | +++ | ++ | - | +/- |
| Euthyroid 0.4 mg progesterone | +++ | +++ | - | +/- |
| Hyperthyroid 2.0 mg progesterone | ++ | + | +++ | +++ |
| Hyperthyroid 0.4 mg progesterone | + | +++ | +/- | +++ |
| Hypothyroid 2.0 mg progesterone | + | + | ++ | ++ |
| Hypothyroid 0.4 mg progesterone | + | +/- | - | - |

Autoradiographs were analyzed for at least five blastocysts in each experimental group.

- +++ = heavy
- ++ = medium
- + = light
- +/- = very light
- = absent

dependent delay period. Possibly this effect was exerted by way of the thyroid hormone influencing the uterus since thyroid hormone was shown to exert an effect upon uterine physiology during the delay period (11). No information had been obtained concerning whether the blastocysts themselves showed metabolic differences in response to the thyroid hormone. Thus, the current studies were conducted to determine whether the maternal thyroid status influences the *in vitro* synthesis of protein and RNA in non-delayed blastocysts and in delayed blastocysts.

The ^{14}C -leucine uptake and incorporation studies with blastocysts which were flushed from the uteri on Day 5 show that the maternal thyroid status does significantly influence protein synthesis in the non-delayed blastocyst. The incorporation of leucine into protein has been used by a number of investigators (e.g. 14, 4, and 17) as a useful means of assessing protein synthesis in preimplantation mammalian embryos. Perhaps in these intact (non-delayed) rats this effect of thyroid hormone on protein synthesis in the blastocyst may help explain the findings of many investigators who have shown hypothyroidism to have detrimental effects upon litter size while thyroid hormone administration was shown to have beneficial effects as reviewed by Leatham (14).

The leucine incorporation and uptake studies were conducted utilizing blastocysts from intact (non-delayed) rats since large numbers of blastocysts were desirable for the protein synthesis studies (experimental delay of implantation leads to a 50% decrease in the number of available blastocysts), and since the diapausing blastocyst has been shown (15) to reduce its metabolic activities. These investigators and Britton-Casimiri et al. (5) demonstrated that diapausing rat blastocysts show increased metabolic activity after 4-6 hours in an appropriate culture medium (removed from uterine influences). For the autoradiographic studies of the incorporation of protein and RNA precursors we utilized delayed blastocysts which were flushed from the uteri on Day 8 and incubated in BMOC-2 for five hours.

This relatively short incubation time does not fully restore the blastocyst to metabolic levels comparable to non-delayed blastocysts. However, it does provide an opportunity to obtain information about blastocysts that are recovering from the progesterone-dependent delay period during which thyroid hormone was demonstrated to exert an effect in our earlier studies. Experiments using various recovery times will be interesting to conduct and are planned, but such blastocysts will be less similar to delayed blastocysts.

Although blastocysts from euthyroid rats with progesterone deficiency showed no differences in the incorporation and distribution of the precursors, the hyperthyroid condition in both progesterone levels did cause an increase in both precursors in the inner cell mass region. The data from the leucine uptake and incorporation studies using non-delayed blastocysts cannot be compared closely with the autoradiographic studies, but the increased protein and RNA synthesis during hyperthyroidism in these delayed blastocysts is in line with the increased protein synthesis seen in the non-delayed blastocysts from hyperthyroid rats. Also in line with the leucine incorporation studies, the autoradiographic studies with blastocysts from hypothyroid rats showed the lowest levels of incorporation of the RNA and protein precursors. The complete absence of the radioactive amino acid and uridine in the trophoblast area of the blastocysts from progesterone-deficient hypothyroid rats may be an indication of a blastocyst condition that contributes to extremely low survival of embryos in rats of this treatment during delayed implantation as reported by Holland et al. (10)

Recently, it was shown (8 and 9) that the preimplantation embryos of the rat and rabbit synthesize steroid hormones; and it has been suggested (9) that these steroid hormones of the blastocyst are critical for preimplantation embryogenesis. It also has been suggested (1) that blastocysts of the rabbit accumulate steroids from the uterine fluid. Some of the evidence that the blastocyst is able to synthesize progesterone and estrogen was obtained by histochemically demonstrating the activity of 3 β hydroxysteroid dehydrogenase, a key enzyme in steroidogenesis. Interestingly, Dickman et al. (9) reported that in their studies with rabbit blastocysts, for most embryos, the 3 β -HSD activity was stronger in the inner cell mass (embryonic disc) than in the trophoblast. In our current experiments, the blastocysts from progesterone deficient hypothyroid rats were the only ones which showed an absence of both protein and RNA precursor incorporation in the inner cell mass and, in delayed implantation studies, this group is the one for which progesterone deficiency is most detrimental. Preliminary studies of 3 β -HSD synthesis in our blastocysts indicate the possibility that progesterone-deficient hypothyroid rats show a lower level of this enzyme in their blastocysts.

Acknowledgements

This research was supported by the National Science Foundation Grant GB-8241 and by a Creative and Research Fellowship from Omega Psi Phi, Inc. to J.P.H.

Literature Cited

1. BORLAND, R. M., G. F. ERICKSON, and T. DUCIBELLA, (1977). Accumulation of steroids in rabbit preimplantation blastocysts. *J. Reprod. Fert.* 49, 219-224.
2. BRINSTER, R. L. (1970). Mammalian embryo culture. In: *The Mammalian Oviduct*, pp. 419-441. Ed. E.S.E. Hafez and R. J. Blandau. The University of Chicago Press, Chicago, Illinois.
3. BRINSTER, R. L. (1971). Uptake and incorporation of amino acids by the preimplantation mouse embryo. *J. Reprod. Fert.* 27, 329-338.
4. BRINSTER, R. L., J. L. WIEBOLD, and S. BRUNNER, (1976). Protein metabolism in preimplantation mouse ovaries. *Devel. Biol.* 51, 215-224.
5. BRITTON-CASIMIRO, V., J. L. BRUN, and A. PSYCHOYOS, (1976). Uptake and incorporation of [3 H] uridine by normal or diapausing rat blastocysts after various periods of culture. *J. Reprod. Fert.* 46, 477-448.
6. COCHRANE, R. G. and R. K. MEYER, (1957). Delayed nidation in the rat induced by progesterone. *Proc. Soc. Exp. Biol. Med.* 96, 155-159.
7. DAENTL, D. L. and C. J. EPSTEIN, (1971). Developmental interrelationships of uridine uptake, nucleotide formation and incorporation into RNA by early mammalian embryos. *Devel. Biol.* 24, 428-442.
8. DEY, S. K. and Z. DICKMAN, (1974). Δ^5 -3 β Hydroxysteroid dehydrogenase activity in rat embryos on days 1 through 7 of pregnancy. *Endocrinology* 95, 321-322.
9. DICKMAN, Z., S. K. DEY, and J. S. GUPTA, (1975). Steroidogenesis in rabbit preimplantation embryos. *Proc. Natl. Acad. Sci.* 72, 298-300.
10. HOLLAND, J. P., J. M. DORSEY, N. N. HARRIS, and F. L. JOHNSON, (1967). Effect of thyroid activity upon delayed implantation of blastocysts in the rat. *J. Reprod. Fert.* 14, 81-85.
11. HOLLAND, J. P., F. L. CALHOUN, N. N. HARRIS, and N. W. WALTON, (1968). Uterine alkaline phosphatase and blastocyst implantation during altered thyroid activity. *Acta Endocr., Copenh.* 59, 335-343.
12. HOLLAND, J. P., J. M. FINLEY, R. D. KAZWELL, and F. L. MESHBERGER, (1970). Progesterone-dependent blastocyst survival during altered thyroid activity in the rat. *J. Reprod. Fert.* 23, 143-146.

13. LEATHEM, J. H. (1972). Role of the thyroid. In: *Reproductive Biology*, pp. 857-876. Eds. H. Balin and S. Glaser. Excerpta Medica, Amsterdam.
14. MONESI, V. and V. SALFI, (1967). Macromolecular synthesis during early development in the mouse embryo. *Exp. Cell Res.* 46, 632-635.
15. RATH, N. C. and A. PSYCHOYOS, (1976). In vitro incorporation of [³H]-thymidine by diapausing rat blastocysts. *J. Reprod. Fert.* 48, 225-227.
16. SKALKO, R. G. (1971). Methods for histologic and autoradiographic analysis of the early mouse embryo. In: *Methods in Mammalian Embryology*, p. 241. Ed. J. C. Daniel, Jr., W. H. Freeman Co., San Francisco, California.
17. TASCA, R. J. and N. HILLMAN, (1970). Effects of actinomycin D and cycloheximide on RNA and protein synthesis in cleavage stage embryos. *Nature, London*, 225, 1022-1025.