UTERINE ALKALINE PHOSPHATASE 
AND BLASTOCYST IMPLANTATION DURING 
ALTERED THYROID ACTIVITY

By 
James P. Holland, F. J. Calhoun, N. N. Harris 
and N. W. Walton

ABSTRACT

Using the technique of delayed implantation of blastocysts in the rat, hyperthyroidism counteracts the effects of progesterone deficiency upon blastocyst survival and implantation while hypothyroidism contributes to the severity of the progesterone deficiency. In non-pregnant rats, treated in a manner to duplicate the delayed implantation experiments, the localization pattern of alkaline phosphatase within the uterine endometrium closely corresponds with the fate of the blastocysts. Hyperthyroidism during progesterone deficiency restores to normal the distribution of uterine alkaline phosphatase while hypothyroidism is associated with a further depletion of uterine alkaline phosphatase. Oxygen consumption studies indicate that the changes in the alkaline phosphatase are not associated with changes in the general uterine metabolism.

Many investigators have shown that the ovarian hormones influence the distribution and concentration of uterine alkaline phosphatase (e.g., Hayashi & Fishman 1961; Atkinson & Elftman 1947; Lobel et al. 1965 a, b; Giering & Zarrow 1958). Although the importance and precise role of this enzyme in reproductive physiology are not clear, it has been implicated in a number of important cellular processes such as transport of materials across cell membranes (Moog 1946), phospholipid synthesis (Malone 1960), and the activity of this enzyme has been shown to increase in relation to ribonucleic acid (RNA) synthesis (Gavasto & Pileri 1958). During normal implantation alkaline phosphatase has been shown by Manning et al. (1966) to increase in the uterus.
between Days 5 and 8 of pregnancy (time span coinciding with implantation) and to be localized at the implantation sites (Lobel et al. 1965a, b). Since recent studies in our laboratory showed that during low progesterone levels, thyroid activity significantly influences the implantation of delayed blastocysts, investigations were initiated to determine how the uterine environment differs during the conditions of altered thyroid activity and progesterone deficiency. As a part of these investigations, experimental conditions simulating those used in the delayed implantation studies were examined for their effect upon uterine alkaline phosphatase activity and upon uterine oxygen consumption.

MATERIALS AND METHODS

The procedure for obtaining the delayed implantation of blastocysts has been described in detail elsewhere (Holland et al. 1967). Briefly, it is based upon the method of Cochrane & Meyer (1957) and involves ovariectomy on Day 3 of pregnancy, daily injection of progesterone through Day 8, verification of delay by laparotomy on Day 8, and supplementing the progesterone with oestrone on Days 9 through 14 to obtain implantation and development of the blastocysts. In the present investigations Holtzman albino rats, between 90 and 120 days old were injected daily, subcutaneously, with 2.0 mg or 0.4 mg progesterone for six days (the sixth day corresponds to the day of laparotomy, Day 8, in the delayed implantation studies). Some of the animals of each dosage level were sacrificed after the six days of progesterone treatment, while in others the daily progesterone dose was supplemented with 1 µg oestrone for an additional two days and the animals were sacrificed 48 hours following the beginning of the oestrone treatment (time corresponding to the time of optimal metrial sensitivity caused by the progesterone-oestrogen sequence and the span during which implantation occurs, according to Psychoyos 1967). Control, hyperthyroid, and hypothyroid rats were tested receiving each progesterone dose, with and without oestrogen supplementary treatment. Hyperthyroidism was induced by subcutaneous injection of 48 µg L-thyroxine/day beginning at least ten days prior to ovariectomy while hypothyroidism was induced by surgical thyroidectomy at least one month prior to ovariectomy. All animals were sacrificed by cervical fracture.

For the alkaline phosphatase studies uterine tissue was immediately removed and quickly frozen at −20°C in a Harris International cryostat microtome. Cross sections were cut at 10 µm, picked up with the slides, and allowed to dry at room temperature for 30–45 minutes. The alkaline phosphatase reaction was accomplished by the Gomori method of calcium-cobalt precipitation (Pearse 1961) using β-glycerophosphate as the substrate. Some uterine sections were also prepared which were exposed to each solution except the substrate, and these served as control slides for the reaction. From these same rats from which tissue for the alkaline phosphatase studies was obtained, additional segments (about 40 mg each) of uterine tissue were removed, slit longitudinally, and placed in Warburg flasks containing 3.0 ml Krebs-Ringer phosphate buffer and gassed with oxygen for determination of the rate of oxygen consumption. Readings were taken at 15 minute intervals for a two-hour period. For these Warburg studies as well as for the alkaline phosphatase studies rats were used which were sacrificed on the equivalent of Day 8 of the delayed implantation experiments and ones which were sacrificed 48 hours after the initiation of oestrogen treatment.
RESULTS

For a more meaningful correlation of the data, Fig. 1 presents a composite of some of the pertinent delayed implantation data aligned with the respective uterine findings associated with the particular progesterone or progesterone-thyroid treatment. It can be seen from the bar graphs in Fig. 1 that the average number of implantation sites occurring in all groups of animals receiving 2 mg progesterone was about equal (5.1–6.1 sites/rat), neither hypernor hypothyroidism exerted an effect. Lowering the daily maintenance dose of progesterone from 2 mg to 0.4 mg causes a significant \( P < 0.05 \) decrease in the number of blastocysts implanting in control animals and a still further decrease in the number implanting in hypothyroid animals. The low progesterone-treated hyperthyroid animals, however, showed a significant \( P < 0.05 \) increase in the number of implanting blastocysts as compared with the low progesterone-treated controls. (Ovulation was not altered as determined by

![Figure 1](https://via.placeholder.com/150)

**Fig. 1.**

The effect of progesterone dosage and alterations of thyroid activity upon delayed implantation of blastocysts, uterine alkaline phosphatase localization, and uterine oxygen consumption in the rat. The numbers in parenthesis represent the number of animals or samples in each study while the vertical lines on the bar graphs and the ± of the oxygen consumption data represent the standard error of the mean.
oviduct flushings in other experiments.) These findings are discussed in detail elsewhere (Holland et al. 1967).

Immediately above the bar graphs of Fig. 1 is shown a tabulation of the intensity of the alkaline phosphatase reaction in the three regions of the uterine endometrium for the various treatment groups used in the delayed implantation studies. In addition, the tabulation in the upper-most portion of Fig. 1 shows the oxygen consumption values in $\mu l/mg/h$ for the uterine tissue from these same animals. It is seen that lowering the daily maintenance dose of progesterone from 2 mg to 0.4 mg also caused a decrease in the levels of alkaline phosphatase in the endometrial epithelium and glands. Corresponding with the increased number of implantation sites shown in the bar graphs, the uteri of the non-pregnant, low progesterone-treated hyperthyroid rats showed a rebound or a restoration of the endometrial alkaline phosphatase to an intensity even greater than that seen in control 2 mg progesterone-treated animals. The uterine alkaline phosphatase findings for rats which received oestrone along with the progesterone for an additional 48 hours are not shown in the tabulation of Fig. 1. However, the oestrone addition was found to increase the intensity of the alkaline phosphatase reaction in each of the three endometrial regions for each group (2 mg and 0.4 mg) of control and hyperthyroid rats to the + + + level (as is seen in the 0.4 mg hyperthyroid tabulation) in the cases which did not already show this level in Fig. 1. On the other hand, hypothyroid rats of both the 2 mg and the 0.4 mg progesterone dose showed only a slight increase in alkaline phosphatase activity following the addition of oestrone. This slight increase occurred in the uterine glands; the uterine epithelium remained negative in all thyroidectomized animals.

The uterine oxygen consumption, shown in the upper-most section of Fig. 1, on the other hand, cannot be closely correlated with the progesterone-thyroid alterations and thus, the changes in the intensity of the alkaline phosphatase reaction cannot be attributed to changes in the general metabolism of the uterus. Control $QO_2$ data showed that the oxygen consumption of the rat uterine slices varies with the stage of the oestrous cycle, being highest in prooestrus ($1.128 \pm 0.055 \mu l/mg/h$) and lowest in dioestrus ($0.868 \pm 0.090 \mu l/mg/h$). This agrees with the findings of other investigators (e.g. Kerly 1937). Also, ovariectomy was found to significantly decrease uterine oxygen consumption ($0.829 \pm 0.037$; $P < 0.01$ when compared with the prooestrus level) and daily administration of 48 $\mu g$ L-thyroxine for 10 days was found to significantly elevate the $QO_2$ of uteri from ovariectomized rats ($0.995 \pm 0.030$; $P < 0.01$ when compared with euthyroid ovariectomized rats) while thyroidectomy was without effect in intact or in ovariectomized rats. In Fig. 1 the significantly higher $QO_2$ of the 0.4 mg control uteri ($0.985 \pm 0.02$) in comparison with the 2 mg controls ($0.826 \pm 0.03$; $P < 0.01$) may indicate suppression of the uterine $QO_2$ by the higher dosage of progesterone since Asdell (1964) reported that
the rat corpora lutea produce about 0.5 mg progesterone/day to neutralize the normally occurring oestrogen of pregnancy. Suppression of tissue QO₂ with high doses of progesterone has been reported by other investigators such as Hassegawa (1959) and Wade & Jones (1956). The other uterine QO₂ changes listed are not statistically significant. In addition to the QO₂ values shown in Fig. 1, oxygen consumption studies were made using uterine tissue from the six listed treatment groups following two days of oestrogen treatment. The supplementary oestrogen treatment caused no significant changes in the oxygen consumption values of any treatment groups.

Fig. 2 shows representative photographs of the alkaline phosphatase reaction in uterine sections from the various treatment groups described above. The photographs on the left (numbers 1, 3, and 5) show sections from 0.4 mg progesterone-treated animals while those on the right are from 2 mg progesterone-treated animals. The top row (pictures number 1 and 2) show control animals, numbers 3 and 4 are from thyroidectomized rats, while numbers 5 and 6 are from hyperthyroid rats. Comparison of photographs number 1 and 2 reveals that the alkaline phosphatase reaction is less in photograph number 1 (the low progesterone-treated control) and the uterine glands (g) which are seen with the higher dose are not present with the low dose. Both photographs from the thyroidectomized animals show reduced glandular epithelium and the positive alkaline phosphatase reaction is confined mainly to the capillaries (c). The reaction is strikingly positive in the hyperthyroid rats of both progesterone treatment groups. The 0.4 mg treated animals show pronounced glandular proliferation with a marked positive reaction and the luminal epithelium (e) is strongly positive. The hyperthyroid condition counteracts the effects of progesterone deficiency upon uterine glands and alkaline phosphatase distribution. Fig. 3 shows: (1) a photograph of the control slide for the reaction, (2) alkaline phosphatase reaction in a section from an ovariectomized (11 day) untreated rat; note the negative reaction in the epithelium and a slight reaction in the glands, and (3) alkaline phosphatase reaction in a normal metoestrus rat. (The vaginal smear showed an equal number of cornified cells and leukocytes.)

**DISCUSSION**

Apparently, for blastocyst implantation and uterine alkaline phosphatase distribution, hyperthyroidism compensates for progesterone deficiency within the dosage levels studied. This may be associated with some effect of thyroid hormone upon the metabolism (utilization or availability) of progesterone. The alkaline phosphatase may be influenced by thyroid hormone directly (studies with uteri from ovariectomized rats without replacement therapy indicate this
Fig. 2.

Histochemical localization of alkaline phosphatase in frozen sections of rat uterine tissue ($\times$ 66) from animals pre-treated in a manner to simulate the studies of thyroidal influences upon delayed implantation. The sections on the left are from 0.4 mg progesterone-treated rats while those on the right are from 2 mg progesterone-treated rats. The top row (photographs 1 and 2) are control animals, the middle row (photographs 3 and 4) are hypothyroid, and the bottom photographs (5 and 6) are hyperthyroid animals. Dark areas indicate a positive alkaline phosphatase reaction. Note differences in the intensity of the reaction in the capillaries (c), epithelium (e), and glands (g) of the various treatments.
Fig. 3.
Histochemical localization of alkaline phosphatase in frozen sections (× 80) of uteri from rats used for control observations. Photograph number 1 shows a section (from a 2 mg progesterone-treated rat) which was taken through each step of the alkaline phosphatase procedure except incubation in the substrate and thus no darkened areas. Photograph number 2 shows the alkaline phosphate reaction in an untreated animal which had been ovariectomized for 11 days while photograph number 3 shows the reaction in a normal metoestrus rat.
possibility) in addition to being influenced by thyroid hormone indirectly by means of a thyroidal-induced alteration of the progesterone metabolism.

Alkaline phosphatase may be influential in the survival and implantation of blastocysts. As pointed out earlier, this enzyme has been found (Manning et al. 1966) to increase in the uterus between Days 5 and 8 of pregnancy, and it has been found (Lobel et al. 1965 a, b) to become localized at the implantation sites. Lobel et al. (1965 a, b) state that increased alkaline phosphatase activity in the uterine capillary endothelial cells suggests increased permeability of the capillary walls. In like manner, the increased activity of this enzyme in the glandular epithelium may be associated with increased secretory activity. These alkaline phosphatase-influenced conditions may be important in the maintenance of a progestational uterine environment for blastocyst survival. The oxygen consumption findings seem to indicate an unlikelihood of a thyroxine-induced alteration of the general uterine metabolism as being the responsible factor for the alkaline phosphatase changes. The differences in uterine alkaline phosphatase (especially in the luminal epithelium which the blastocyst contacts directly) may be associated with differences in the transport of essential nutriments and/or metabolites for the delayed blastocysts.

The decreased numbers of blastocysts which implanted following the delay period in progesterone deficient rats of the delayed implantation studies, probably resulted primarily from failure of the blastocysts to survive during the progesterone-dependant maintenance period of the delay (i.e., Days 3–8). In such a case, the increased numbers obtained in hyperthyroid-progesterone-deficient rats results from a thyroidal effect upon the progesterone-dependant maintenance rather than an effect upon the oestrogen-dependant implantation stimulus. Meyer & Nutting (1964) concluded that once the blastocyst reaches the lower portion of the oviduct or the lumen of the uterus, its continuing survival is dependant mainly on the quantity of progesterone available. They say that according to their results with delayed implantation in rats, progesterone appears to be vitally concerned with maintaining the viability of the blastocysts during the pre-implantation period. Preliminary findings in our laboratory from oviduct and uterine flushings support this idea since the number of blastocysts which can be obtained from uteri flushed on Day 8 is lower in progesterone deficient rats than in controls.

Concerning the possibility of a thyroxine-induced alteration of the progesterone metabolism, our preliminary findings using 4-14C-progesterone in a modification of the procedure of Short & Rowell (1963) show that hyperthyroid rats have significantly lower blood levels of progesterone after the same time interval when compared with euthyroid rats. Possibly the hyperthyroid condition is promoting an increased metabolism and/or utilization of progesterone which may be advantageous to the economical or efficient use of low levels of progesterone. The action of thyroxine in the augmentation of the progesterone
effect upon uterine alkaline phosphatase may be an important intermediary by means of which progesterone influences blastocyst survival and implantation. Further studies of progesterone metabolism and determinations of the biological half-life of progesterone are being conducted to aid in identifying the influential processes.

ACKNOWLEDGEMENT

This work was supported by NIH grant HD 01076 from the Institute of Child Health and Human Development.

REFERENCES


Received on February 2nd, 1968.