ACTION OF THYROXIN ON DEVELOPMENT OF HIND LIMB BONES OF MOUSE EMBRYOS OF MUTANT LINE BRACHYPODISM-H

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It has been shown that thyroxin (T_4) and triiodothyronine (T_3) influence the growth and differentiation of anlagen of the long bones of chick embryos in vitro [3, 4, 7]. Triiodothyronine stimulated growth of slowly growing bones – the radius and ulna, but inhibited growth of rapidly growing bones – the femur and tibia [8]. The effect of T_3 on bone growth in vitro has been found [9] to depend on the concentration of the hormone in the culture medium.

The effects of the brachypodism (bp) gene in mice have been described [6]. The limbs of homozygotes (bp bp) are shortened, the hind limbs more so than the forelimbs. The bones of the hand and foot also are much shorter than normally.

In mouse embryos homozygotic relative to the brachypodism-H gene (bp^H), growth and differentiation of the long bones of the limbs, especially the fibula, are retarded [2]. Some doses of T_3 stimulate growth of the fibula of 13-day bp^H bp^H embryos in vitro [1].

The object of this investigation was to study the action of thyroxin in vivo on growth and differentiation of bones of the hind limb in bp^H bp^H mouse embryos.

EXPERIMENTAL METHOD

Experiments were carried out on mice of the mutant line brachypodism-H, discovered in Harwell (England) as a repeated mutation of the brachypodism gene. Mice of this mutant line for these experiments were obtained from Jackson's laboratory (USA).

Females (bp^H bp^H) were crossed with bp^H bp^H males, and starting on the 11th day of pregnancy the females received intraperitoneal injections of L-thyroxin (Koch–Light laboratories). The zero day of pregnancy was taken as the day when a vaginal plug was found. The thyroxin was dissolved in 0.001N KOH

TABLE 1. Number and Length of 17-Day bp^H bp^H Embryos after Injection of Thyroxin

Series of experiments	Dose of T_4 (in μg/g body weight (in days)	Period of injection of T_4 (in days)	No. of pregnant females	No. of implantations	No. of embryos: absorbed, absolute	absorbed, %	absolute, absol.	absolute, %	Living	Length of embryos: in mm	in % of control
I	1	11—14	20	144	7	4,8	0		137	19,3±0,11†	102,1
II	2	11—14	22	164	30	18,2	1	0,6	133	19,4±0,09	102,8
III	4	11—14	21	147	18	12,2	2	1,4	127	19,7±0,10	104,6
IV	8	11—14	9	69	30	43,4	5	7,2	34	20,0±0,18	106,2
V	2	11—16	16	118	24	20,3	11	9,3	83	19,4±0,12	102,9
VI	4—2—1*	11—16	14	89	13	14,6	7	7,9	69	19,7±0,16	104,5
Control	0	—	16	127	6	4,7	1	0,8	120	18,8±0,13	100

* 4 μg injected on 11th-12th day, 2 μg on 13th-14th day, and 1 μg on 15th-16 day.

† $P = 0.02$; in other cases $P < 0.01$.

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TABLE 2. Dimensions of Bones of Hind Limb of 17-Day $bp^H bp^H$ Embryos after Injection of Thyroxin

Series of experiments	Dose of T_4 (in mg/g body weight)	Period of injection of T_4 (in days)	Femur			Tibia			Fibula			Foot	
			n	length in mm	length in % of control	n	length in mm	length in % of control	n	length in mm	length in % of control	n	length in mm	length in % of control
I	1	11—14	137	2,45±0,03	114,0	137	2,85±0,02	107,0	136	1,39±0,02	108,5	133	3,72±0,03	106,0
II	2	11—14	129	2,37±0,03	110,0	127	2,58±0,02	96,0	128	1,51±0,03	118,0	117	3,72±0,02	106,0
III	4	11—14	127	2,53±0,02	117,5	127	2,92±0,02	109,0	127	1,41±0,02	110,0	121	3,94±0,03	112,5
IV	8	11—14	34	2,21±0,03†	103,0	34	2,66±0,02†	100,0	34	1,21±0,02†	95,0	34	3,65±0,04	104,3
V	2	11—16	83	2,53±0,03	117,5	83	3,06±0,03	114,4	83	1,46±0,03	114,0	77	3,77±0,03	107,5
VI	4—2—1*	11—16	68	2,55±0,03	118,6	68	2,93±0,03	109,7	68	1,42±0,02	110,9	63	3,73±0,04	106,5
Control	0	—	120	2,15±0,02	100	120	2,67±0,02	100	120	1,28±0,03	100	117	3,50±0,02	100

* 4 μg injected on 11th-12th day, 2 μg on 13th-14th day, and 1 μg on 15th-16th day.

† Differences from control not statistically significant, $P > 0.05$; in other cases $P < 0.01$.

and injected in a volume of 0.1 ml. The following doses of T_4 were used: 1, 2, 4, and 8 μg/g body weight. Control pregnant females received an injection of the same volume of solvent. After the 17th day of pregnancy, the females were autopsied and the number of implantations and the numbers of absorbed, dead, and living embryos were determined. Embryos dying in the early stages of development were regarded as absorbed, and embryos dying between 15 and 17 days were classed as dead. The living embryos were weighed and their crown-rump length measured. The length of the limb bones was measured in preparations cleared and stained with alizarin by Green's method [5]. Because the hind limbs are more severely affected than the forelimbs in bp^H bp^H mice, the bones of the hind limbs only were measured. The bones were drawn by means of a type RA-4 drawing apparatus under a binocular microscope (linear magnification 30 ×). When the length of the bone was measured allowance was made for its curvature. The foot was measured from the ankle joint to the end of the distal phalanx of the 3rd toe. The degree of ossification of the bone was determined from the size of the cuff of bone. For histologic examination the bones were fixed in Zenker's solution and sections were stained with hematoxylin-eosin.

EXPERIMENTAL RESULTS

Injection of T_4 in the course of 11-14 days of pregnancy led in all series of experiments to an increase in length of the embryos, the increase being greater with the larger doses of hormone. Injection of T_4 in a dose of 2 and 4 μg/g body weight was accompanied by an increase in the number of absorbed embryos, while in a dose of 8 μg/g body weight it led to absorption of 43.4% of embryos and to a considerable increase in the number of dead embryos (Table 1).

The results given in Table 2 show that a significant increase in length of all bones of the hind limb took place in the embryos of the experiments of series I, II, and III with the exception of the tibia in the experiments of series II. The data showing changes in size of the bones in the experiments of series II differ appreciably from the results of series I and III. The length of the fibula in the experiments of series II was greater by a statistically significant margin than the length of this bone in series I and III. At the same time, the length of the femur was less in the experiments of series II than its length in series III ($P < 0.001$), while the tibia was actually shorter than in the control.

Injection of a large dose of hormone (series IV) did not lead to a significant change in length of the femur, tibia, or fibula by comparison with the control. In this series of experiments a significant increase in size of the foot only was observed. The absence of increase in size of the long bones in the experiments of series IV was evidently due to the fact that this dose of hormone is slightly toxic. Further evidence of this was given by the increased mortality of the embryos (Table 1).

As was mentioned above, the fibula was most severely affected in the bp^H bp^H embryos. For this reason, the fact that the greatest increase in size of the fibula took place in the experiments of series II shows that this dose of hormone is optimal

as regards its effect on growth of this bone. It was considered that prolonging the period of injection of T_4 in a dose of 2 μg/g body weight may lead to a return even closer to normal growth of the fibula. It was found that administration of the hormone in this way (series V) caused a sharp increase in the percentage of dead embryos, which was about six times greater than in the experiments of series III and was actually higher than the percentage of dead embryos in series IV (Table 1). As was mentioned above, the dose of T_4 used in the experiments of series IV was toxic and did not lead to any change in the size of the limb bones by comparison with the controls. At the same time, the size of the limb bones in the embryos of series V increased considerably, the degree of enlargement of all the bones being about the same (Table 2).

The increase in the percentage of dead embryos in the experiments of series V was connected with their death in the terminal periods of injection of the hormone. This was evidently the result of increased permeability of the placenta in the late stages of development, so that a large amount of hormone enters the embryo's blood stream. For this reason it was decided to use different doses of hormone in the course of 11-16 days of pregnancy—4, 2, and 1 μg/g body weight. Each of these doses was injected into the same animal in the course of 2 days of pregnancy (series VI). Administration of the hormone in this way did not cause any increase in growth of the bones by comparison with that observed in the experiments of series V (Table 2). However, the number of dead embryos in the experiments of series VI was slightly smaller (Table 1).

Consequently, injection of the hormone was most effective in a dose of 4 μg/g body weight in the course of the 11th-14th days of pregnancy (series III). In the other series of experiments, increased mortality of the embryos or a less marked stimulant action of T_4 on growth of the limb bones was observed.

Administration of T_4 accelerated the process of ossification of the femur in all series of experiments except IV. The degree of ossification of the tibia in series I, III, V, and VI was greater than in the control. T_4 had no appreciable effect on ossification of the fibula, although in the experiments of series II more bones with a "bone cuff" were observed than in the control. In series IV the ossification of the tibia and fibula was slightly retarded, indicating the toxic action of the hormone in a dose of 8 μg/g body weight.

The histologic study of the femur and fibula revealed a greater degree of hypertrophy of the chondrocytes in the experimental embryos than in the controls.

As a result of administration of T_4 the increase in length of the limb bones (in %) was approximately twice or three times greater than the increase in length of the embryos, by comparison with these indices in the controls (Tables 1 and 2). The results obtained demonstrate that T_4 has a more marked stimulant effect on growth of the affected limb bones than on growth of the axial skeleton, development of which is undisturbed in bp^H bp^H mice.

The increase in length of 17-day embryos as a result of administration of T_4 in a dose of 8 μg/g body weight is evidently associated with the increased mortality among the smaller embryos resulting from the action of this toxic dose of the hormone. The fact that the dimensions of the long bones of the limbs in the embryos of series IV do not differ statistically significantly from the controls indicates possible suppression of their growth.

Hence, injection of T_4 in the course of the 11th-14th or 11th-16th days of pregnancy leads to specific stimulation of growth of the limb bones in bp^H bp^H embryos, and the most marked normalizing effect of the hormone on growth of bones with inherited lesions is observed if it is injected during the 11th-14th day of pregnancy in a dose of 4 μg/g body weight.

According to results obtained by some workers, injection of T_4 stimulates the synthesis of chondroitin-sulfuric acid in the skin of rats [10]. The results obtained in the present investigation, and also results of experiments in which the action of T_3 was studied on growth of bone anlagen in vitro [1], show that thyroid hormones increase the hypertrophy of the chondrocytes in affected bones of bp^H bp^H embryos. It may be postulated that thyroid hormones stimulate the synthesis of acid mucopolysaccharides in the chondrocytes of these bones, which leads to an increase in their growth.

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