FROM THE ORTHOPAEDIC DEPARTMENT, UNIVERSITY OF UPPSALA, SWEDEN.
FORMER CHIEF: ACTING PROF. J. A. SEVASTIKOGLOU, M.D.

INSULIN INDUCED MICROMELIA IN CHICKENS

I. *Morphological Study*

By

JOHN A. SEVASTIKOGLOU, M.D.

Since the early days of medicine congenital malformations have always attracted attention. Interest in the study of these conditions has, however, grown progressively since the beginning of the 20th century when statistical reports on the occurence of congenital abnormalities, and the correlation between their frequency and various pathological affections of the mother organism during gestation demonstrated an unbelievably high frequency of these conditions. Furthermore, the development of the new methods of investigation during the last decades has greatly increased the possibilities of studying the problem from different aspects.

Experimental teratogenesis is one of the methods of biological investigation more frequently used for the study of congenital malformations. The application of this method for more than one century has added a great deal to our knowledge regarding the morphological development and the influence of different environmental factors on the production of these abnormal conditions. Mechanical, X-ray, anoxic, chemical and other factors applied to pregnant animals or fertilized hens' eggs have been proved to give a teratogenetic response.

In a large series of experiments *Landauer* (1945) found that insulin injected into the yolk sac of fertilized hens' eggs before or during the early stages of incubation, had a teratogenetic effect inducing rumplessness. Injection at later stages of incubation was responsible for deformities of the beak, the extremities and the eyes (*Landauer*, 1947). During the following years Landauer and his co-workers published several works on the teratogenetic properties of insulin. Several other investigators (*Duraiswami*, 1952, *Zwilling*, 1952, *Anderson et al.*, 1959, and others) later confirmed the observations made by Landauer. Other

substances were also proved to induce similar developmental malformations when injected into eggs. An extensive list of references to the early investigations can be found in the work of *Duraiswami* (1952).

The experience gained by these and numerous other works on experimental teratogenesis can be summarized by the statement recently made by *Hamilton* (1960) that a) there is a multiplicity of agents that can produce abnormalities, b) the time factor when the particular agent is administered is of importance and c) that early embryonic tissue is susceptible to particular noxious agents.

The present investigation has been undertaken to study and compare the biochemical composition of the skeleton during the embryonic development of normal and insulin-induced micromelic chicks. Morphological and histological studies of the material have also been performed. The purpose of this work is to look for correlation between the morphological—histological changes and the biochemical composition of the skeleton during normal and teratogenic embryonic development. In this paper the morphological observations are described and discussed.

MATERIALS AND METHODS

Insulin was injected into 1094 eggs, a physiological saline solution into 40, while 741 untreated fertilized eggs were incubated and served as controls.

The injection technique used was as follows: the egg shell was carefully washed, first with an antiseptic solution, then with alcohol and it was opened on the air chamber with sterile forceps. Using good illumination, the embryo and the vessels of the vascular area were easily seen. Using a tuberculin syringe a very fine hypodermic needle was driven through the inner layer of the shell membrane into the yolk sac avoiding any injury of the embryo and the vessels. The insulin or saline solution was then injected very slowly. The needle was carefully withdrawn and the egg was coated with a sheet of sterile aluminium foil and placed in the incubator at 37° C. The temperature was never found to exceed the range of ± 0.5° C.

Crystalline insulin insulin was employed.[1] On the day of injection an insulin solution in physiological saline was prepared, in concentration of 5 mg. or 120 I.U./ml. Since crystalline insulin is insoluble at room

[1] The insulin used was kindly delivered by the Pharmaceutical Co "Vitrum", Stockholm, as 7 times crystallized pure powder substance.

temperature at neutral pH, the solution was acidified by adding 3–4 drops in 1n HCL per 10 ml. volume. In this way a clear solution was obtained with a pH between 3.9 and 4.1. In a control series of 234 eggs insulin solution was made in a phosphate buffer with pH of 7.4. Before use the solutions were passed through a sterile glass filter (G5).

The dose of insulin injected in all the cases was 6 I.U., i.e. 0.25 mg. crystalline insulin or 0.05 ml. of the solution per egg.

All eggs were injected 96 hours after incubation and material was collected by opening a certain number of eggs at 24 hours interval up to the time of hatching. Embryos found dead at opening were discarded.

From the material collected every day both tibiae were dissected free from soft tissue and were stored either by deep freezing at —15° C, for biochemical analysis, or in a 10 per cent neutral formaldehyde solution for histological sectioning.

Morphological studies of the occurrence of micromelia were based mainly on measurements of the length of the freshly prepared tibiae and by weighing each bone after thawing before the performance of the chemical analysis. As the purpose of the present investigation was to study insulin-induced micromelia, no systematic record of other deformities in the embryos were made.

Histological studies were based on serial sections of the specimens in 3 μ thick slices, using haematoxylin-eosin, toluidine blue and Azan stainings.

RESULTS

Of the total of 1094 eggs injected with insulin, 215 embryos survived the injection. The average mortality rate in the various insulin-injected series was 80.3 $\pm$1.2 per cent. In the 40 saline-injected eggs the mortality was 70.0 $\pm$7.3 per cent. The mortality rate between eggs injected with acid and neutral insulin solution was 77.4 $\pm$1.5 and 91.0 $\pm$1.9 per cent respectively, the difference being highly significant. Among the 741 fertilized eggs used as controls, the mortality observed was 18.4 $\pm$1.5 per cent.

Morphological studies: Macropscopical observations on general deformities present both in normal and treated specimens were possible only after the 7th or 8th day of incubation. Until this stage of development the embryos are very small and transparent so that any anomalies in

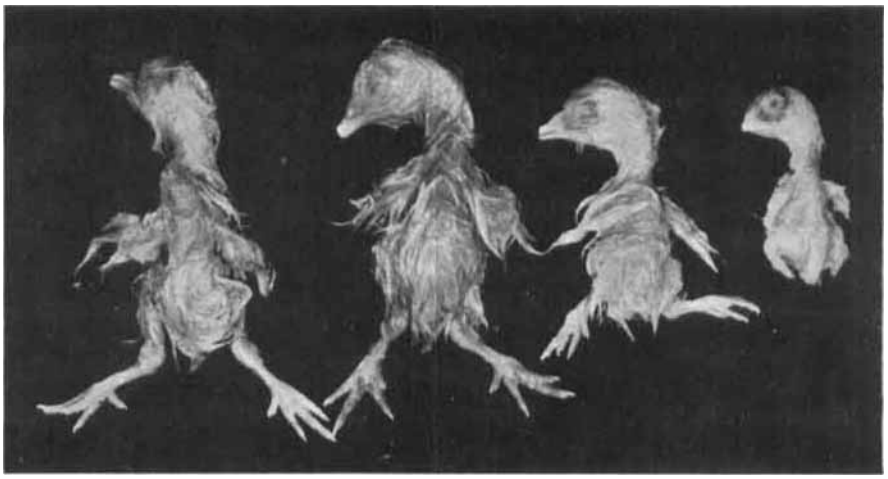

Fig. 1.
17-day old chicken embryos. From the left: a) normal, b) injected with physiological saline, c) insulin-injected with marked micromelia, but without other apparent abnormalities and d) insulin-injected with extreme micromelia and multiple abnormalities of the chondrodystrophic type.

their development are difficult to observe. After this period of incubation anomalies such as anencephaly, anophthalmos, ectopic viscera, club feet etc., can easily be distinguished. Such deformities were often observed in the insulin-injected material. No deformity was observed in the 12 surviving animals injected with physiological saline and opened the 17th day after incubation. As mentioned earlier, no records were made of the frequency of such anomalies. Malformed embryos present among the normal material were discarded. Deformed embryos were included in the injected material.

Compared to the normal controls the insulin-injected embryos displayed two different types of abnormality. In the majority of the cases the embryos were appreciably smaller than normal but as far as it was possible to ascertain they were of the same stage of development and showed no generalized abnormalities. The second type of abnormal development was found in certain embryos which had a monstrous appearance with a very small size, distorted extremities, head and beak deformity, joint stiffness reminding arthrogryphosis and oedematous soft tissues. (Fig. 1). The dissection of the tibiae in this group of deformed embryos was especially difficult as the bones were soft and fragile and the soft tissues were tightly attached to the bones.

The occurrence of micromelia in general, was obvious in every case of insulin-injected embryos. The tibial length was found to be less than the average of the values for normal bones of the same age of incubation in every case after the 8th day of incubation. The ratio of micromelia to highly deformed embryos was 2:1 as was found from the study of 91 insulin-injected tibiae.

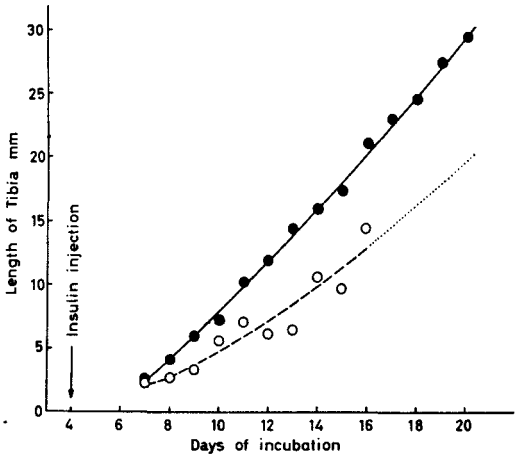

Fig. 2.

The course of the average tibial length during the embryonic development in normal (continuous line) and insulin-treated (broken line) material. The dotted line is based on the statistical extrapolation of the values obtained in the insulin-treated material in comparison to the normal.

The tibial length was recorded between the 7th day of incubation and hatching in the normal material and between the 7th and 16th days of incubation in the insulin-injected embryos. The curve of tibial length of insulin-injected embryos compared to that of normal material showed that after the 8th and up to the 16th day of incubation there was an obvious divergence in the two curves, the insulin treated tibiae being clearly shorter than the normal. A rightly statistically significant difference between the average tibial length in normal and insulin-treated material appeared first the 8th day of incubation. The insulin-treated material was insufficient for observations to be made after the 16th day of incubation. The statistical extrapolation of the curve, however, showed that increasing divergence of the curves continued up to the time of hatching. The course of both curves during the period of incubation followed a regular linear rise. (Fig. 2).

The wet weight of the tibia was studied between the 8th and 20th days of incubation in the normal and between the 8th and 17th days of incubation in insulin-treated material. The tibial weight of insulin-injected embryos showed no statistical difference from the normal between the 8th and 10th days of incubation. Statistically significant differences were, however, obtained from the 11th day of incubation and thereafter, the difference increasing progressively up to the 17th day of incubation. Statistical extrapolation showed that increasing divergence of the curves continued until the hatching. The course of the mean tibial weight values in insulin-treated and normal material followed in both the cases regular parabolic curves. (Fig. 3).

Histological study: The observations reported here are based on sectioned material from a) 4 tibiae obtained from control untreated embryos at the 15th and 17th days of incubation respectively, b) 4 tibiae from embryos at the 17th day of incubation injected with physiological saline, c) 2 tibiae at the 9th day of incubation from insulin-injected embryos, d) 4 tibiae at the 17th day of incubation from insulin-injected micromelic embryos without other anomalies and e) 4 tibiae from insulin-injected embryos with generalized skeletal deformities, as mentioned above, from the 15th and the 17th days of incubation respectively.

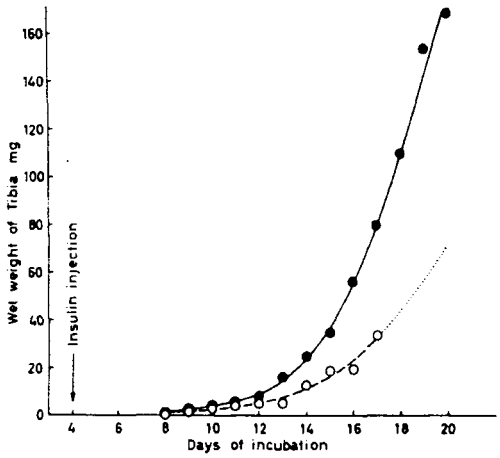

Fig. 3.

The course of the average tibial wet weight during the embryonic development in normal (continuous line) and insulin-treated (broken line) material. The dotted line is based on the statistical extrapolation of the values obtained in the insulin-treated material in comparison to the normal.

The periosteal ossification of the tibia in the normal chick begins at about the 9th day of incubation. A thin bony ring appears first at the middle of the diaphysis, while the remainder of the diaphysis as well as both epiphyses are composed of cartilage disposed in characteristic layers. After this the ring elongates at the epiphyses and the characteristic bony cylinder of the long bones develops in this way.

The 9 day old tibiae of insulin-injected embryos were almost exclusively composed of cartilage and they did not show any deformity or abnormality. In some places at the middle of the shaft signs of primitive bone trabeculae could be identified by Azan staining. The insulin-injected tibiae had much the same structure as normal tibiae at the same stage of development. The metachromatic staining of the cartilage was in the insulin-treated tibiae less pronounced than in the controls. In both cases it was, however, uniform through all the extent of the sections.

The 17 day old tibiae from physiologic saline-injected embryos and the 17th day tibiae from insulin-injected embryos which did not show skeletal abnormalities other than micromelia showed no significant differences in morphology, metachromatic staining and the grade of development from normal bones of the same age.

The 15 and 17 day old tibiae of the insulin-injected embryos, which except for an extreme dwarfism showed generalized skeletal deformities too, showed several structural differences compared to the normal bones of 15 and 17 days of incubation. The zone of flattened chondrocytes of the epiphyseal cartilage cone corresponding to the resting cell cartilage of the mammals was narrower and in some epiphyses almost missing, while the structure of the rest of the cartilage cone was irregular. The hypertrophied cartilage cells were somewhat more numerous and showed a slight increase in their metachromatic staining. The periosteal bone of the diaphysis was significantly less than normal and the bony trabeculae were thinner and more irregular. The diaphyseal bony cylinder was appreciately shorter, while the height of the cones was almost the same as in the normal tibiae. The bone as a whole was thicker and the epiphyses were larger than in the normal. A striking difference between the insulin-injected tibiae of this group and normal bones was that the former showed a strong bending of the bone, localized in the lower third of the diaphysis. The bending, present in all the examined tibiae, exceeded in several cases the 90° angle. The diaphyseal bony trabeculae showed a special arrangement at the site of the bending. There were no signs of fresh or healed fracture at the site of an-

gulation. A marked thickening of the periosteum was present at the concavity of the angulation. In this group of specimens the metachromatic staining of the insulin-treated material was much less pronounced than the specimens of the control group.

DISCUSSION

The total mortality rate among the insulin-injected embryos in the present material was 80.3 ±1.2 per cent and this was considerably higher than in other reports. In *Landauer's* (1947) series mortality rates of 25.7 and 48.8 per cent were reported after injection of 2 or 5 I.U. insulin respectively at the 96th hour of incubation. *Anderson and co-workers* (1959) found in their series a mortality rate of 31.1 per cent by injection of 3–6 I.U. insulin between the 72nd and the 120th hours of incubation. The high mortality rate in the present material was first attributed to the acid pH of the injected solution. However, as already pointed out above, in comparative experiments using acid and neutral insulin solutions the mortality rate was found significantly higher in eggs injected by the neutral solution. The applied technique of injection might to some extent be responsible for the high mortality, since other authors use a somewhat different technique of injection. It can also be assumed that the seasonal quality of the eggs, which has been proved to influence the mortality rate of the embryos, as well as the appearance of spontaneous occurring deformities (*Hutt & Greenwood,* 1928) was a contributing factor to the observed mortality.

Considering the averages of the tibial length, micromelia in insulin-treated material occurred in every case after the 8th day of incubation. *Landauer* (1947) found single micromelia at a maximum of 87.3 per cent, when 5 I.U. insulin were injected at 120 hours of incubation. This frequency was decreased if insulin was injected at earlier or later stages of embryonic development. *Anderson and co-workers* (1959) found on the other hand a frequency of generalized dwarfism in 31 per cent among the injected material of their investigation. The ratio of micromelia to highly deformed embryos was, in the present material 2:1.

The existence of a congenital abnormality in the chicken, resembling the condition known in mammals as "chondrodystrophia foetalis" has been reported first by *Landauer & Dunn* in 1925 and later by *Hutt* (1928) and *Hutt & Greenwood* (1928). *Landauer* in 1947 was able to induce such developmental abnormalities in chickens by injecting insulin in the yolk sac at about the 4th day of incubation. The morpho-

logical and histological appearances of the spontaneously occurring and the insulin-induced anomalies were strikingly similar. These results of *Landauer* have been confirmed later by *Zwilling* (1952). Both these authors found in their material a variation of the degree of the induced micromelia ranging from a slight shortening of the extremities to advanced and multiple skeletal deformities which could be classified as chondrodystrophia.

The results reported in the present communication are on the whole in agreement with those reported by *Landauer* and *Zwilling*. The findings, however, showed that there were two distinctly different skeletal abnormalities induced by the insulin injection, i.e. 1) a mere shortening of the legs of the insulin-injected embryos without other apparent morphological or histological divergences from the normal bone and 2) an extreme shortening of the legs with important histological changes of the bone, combined with other generalized malformations and manifest skeletal deformities. Skeletal deformities of both types appeared in every case in the insulin-treated material. However, statistical evidence provided from the estimations of the length and wet weight of the tibia from control and insulin-injected material show that micromelia appears first between the 8th and 11th days of incubation, i.e. 4 to 7 days after the injection of insulin in the eggs. In the light of these observations, not mentioned by earlier authors, it might be assumed that insulin interacts in some of the early stages of bone formation. It is known (*Fell* 1925) that periosteal bone is laid down in the cartilagenous extremities at about the 9th day of incubation. The effect of insulin on osteogenesis might result in an irreversible distortion of the course of the process, followed by the development of the skeletal deformities described.

SUMMARY

Injection of 6 I.U. insulin in hen's eggs at the 4th day of incubation induces skeletal abnormalities of two main types, i.e. single micromelia without other manifest malformations and extreme dwarfism combined with other generalized and skeletal deformities of the chondrodystrophic type.

Estimations of the length and the wet weight of the tibia in control and insulin-injected material provided statistical evidence that the effect of insulin develops first between the 8th and 11th days of incubation, i.e. 4 to 7 days after the injection.

RESUME

L'injection de 6 u.i. d'insuline dans des oeufs de poules le 4ème jour de l'incubation provoque des anomalies squelettiques de deux types principaux, à savoir micromélie simple sans malformations manifestes et nanisme extrême combiné à d'autres déformités généralisées et squelettiques du type chondrodystrophique.

Les évaluations faites concernant la longueur et le poids humide du tibia dans le matériel de contrôle et le matériel d'observation auquel il a été administré de l'insuline ont fourni la preuve statistique que l'effet de l'insuline ne se développe qu'entre le 8ème et le 11ème jour de l'incubation, c'est-à-dire entre 4 et 7 jours après l'injection.

ZUSAMMENFASSUNG

Injektion von 6 I.U.Insulin in ein Hühnerei am 4ten Tage der Ausbrütung verursacht Skelettabnormitäten von zweierlei Haupttypen, i.e. einfache Mikromelie ohne andere manifeste Missbildungen und extremen Zwergwuchs kombiniert mit anderen generalisierten und Skelettmissbildungen der chondrodystrophischen Art.

Schätzungen der Länge und des Nassgewichtes der Tibia von Kontrol- und insulininjiziertem Material ergab statistischen Beweis dafür, dass die Insulinworkung erst zwischen dem 8. und 11. Brütungstag also 4 bis 7 Tage nach der Injektion auftritt.

REFERENCES

Anderson, C. E., Crane, J. I. & Harper, H. A.: Alterations in Growth-Cartilage in Experimental Dwarfism I. Studies on Insulin-Dwarfed chicks. J. Bone & Joint Surg., *49-A*: 1094, 1959.

Duraiswami, P. K.: Experimental causation of congenital skeletal defects and its significance in Orthopaedic Surgery. J. Bone & Joint Surg., *34-B*: 646, 1952.

Hamilton, W. J.: In Ciba Foundation Symposium on Congenital Malformations, J. and A. Churchill Ltd., London, 1960.

Hutt, F. B. (1927): Studies in Embryonic Mortality in the Fowl. I. The Frequencies of various Malpositions of the Chick Embryo and their Significance. Proc. Roy. Soc. Edinburgh, *49*: 118, 1928/26.

Hutt, F. B. & Greenwood, A. W.: Studies in Embryonic Mortality in the Fowl. II. Chondrodystrophy in the Chick. Proc. Roy. Soc. Edinburgh, *49*: 131, 1925/26.

Landauer, W. & Dunn, L. C.: Chondrodystrophia in Chicken Embryos. Proc. Soc. Exptl. Biol. Med., 23: 562, 1925/26.

Landauer, W.: Rumplessness of Chicken Embryos Produced by the Injection of Insulin and other Chemicals. J. Exptl. Zool., *98*: 65, 1945 p. 291, J. & A. Churchill Ltd., London, 1960.

Landauer, W.: Insulin-Induced Abnormalities of Beak, Extremities and Eyes in Chickens. J. Exptl. Zool., *105*: 145, 1947.

Zwilling, E.: The effect of some Hormones on Development. An. N.Y.Acad. Sciences, *55*: 196, 1952.