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The effect of thalidomide on fetal development and adult testes in Swiss-Webster mice

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ABSTRACT

BIOLOGY

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The Effect of Thalidomide on Fetal Development and Adult Testes in Swiss-Webster Mice

Adviser: Dr. Mary L. Reddick

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Fetal abnormalities were experimentally produced in Swiss-Webster mice by injections of thalidomide, 60 mg/kg of body weight daily. Deformities were manifested in progeny of thalidomide injected males and females.

Evidence from this investigation indicates that the most frequent abnormalities observed were deformities of the limb, although deformities of the mouth, ear, tail, and pelvic region were also observed. The frequency and severity of the abnormalities were definitely influenced by the time at which the injections were given.

Gross analyses revealed that the greatest number of deformities were observed in progeny whose mothers had received injections of thalidomide prior to mating. This fact was an indication that an accumulation of the teratogen was acquired before the critical period of paturition.

Histological studies of the testes from slides prepared by the paraffin method and stained with hematoxylin and eosin revealed that thalidomide affects the formation of sperms.
by: destroying the cells of Leydig, the intercellular matrix, and causing developmental arrest of the spermatogonia.
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CHAPTER I

INTRODUCTION

The production of fetal abnormalities by the administration of tranquillizers and sedatives has provoked much interest. These drug-induced abnormalities mimic to a great degree many of the congenital malformations observed in humans within the present decade.

At a conference in England, Lutwak-Mann (1964) presented data which indicated that men as well as women who take the tranquillizing drug, thalidomide, could risk deformities in their offsprings. The findings of Lutwak-Mann stimulated this investigator to experimentally produce deformities in young mice as a result of both parents having received injections of thalidomide prior to mating. The occurrence of these deformities suggested that the abnormalities may have been due to defects in the ova as well as in the sperms. Evidence from this investigation, however, revealed no changes in the ova. There were definite indications that the malformations of the progeny sired by thalidomide injected males were due to disturbances in the normal formation of the sperms.
CHAPTER II

REVIEW OF LITERATURE

Within the present decade, experimental work on laboratory animals has revealed that a large number of clinical substances have the power, when administered to pregnant females, of producing congenital malformations in the offspring. According to Cahen (1964), some drugs cross the placental barrier and are toxic to the fetus, but do not produce malformations in the developing embryos. Other drugs, however, cause congenital malformation in animals, if given in massive doses, but are of little teratogenic risk to man.

In a retrospective clinical investigation by McBride (1962), it was concluded that a supposedly harmless drug, thalidomide was responsible for fetal abnormalities in patients who had taken small doses of the drug for a short period during the early days of pregnancy. Since Somer's (1960) routine toxicity test conducted in rats revealed no malformations, this conclusion was sharply challenged. As early as 1960, Taussig (1962), reported that the incidence of grossly deformed infants exhibited at a meeting of pediatricians in West Germany, could be traced to thalidomide. Her statement in conjunction with the exhibit attracted little attention.

A few cases of phocomelia, long known as a rare malformation, were observed in Germany in 1959. The number of cases of congenital malformations increased. Since the
latter part of 1961, the number of such cases seen in the United Kingdom, Australia and Europe, reached almost epidemic proportions. These malformations were characterized by amelia, phocomelia and alimentary abnormalities. The common factor appeared to have been the administration of thalidomide during early pregnancy. In cases in which the date of conception was known, the period of sensitivity appeared to be from the twenty-eighth to the forty-second day, (DiPaolo, Gatzek and Pickren, 1964).

Among the various malformations, limb deformities are the most striking (Knapp, Lenz and Nowack, 1962, Luers 1962, and Taussig 1962). The most common dysmelia malformations are: phocomelia, amelia, hypogenesis of the long bones, syndactyly, polydactyly and microphthalmia. Anomalies of the heart, duodenal atresia, absence of the common bile duct and imperfect anuses have been reported.

Evidence indicating that thalidomide does cause phocomelia and a variety of congenital malformations in the growing embryo was manifested by the withdrawal of the drug and failure of new cases to occur. Further proof that thalidomide produces several teratogenic effects in mice was indicated by DiPaolo (1963). He made gross analyses of the litters of 62 pregnant strain A mice which had been given thalidomide orally. According to DiPaola, thalidomide treated animals having litters with deformities appeared to have fewer fetuses than did control mothers, which averaged nine fetuses.
Experiments by Giroud, Tuchmann-Duplessis and Mercier (1962) were carried out on pregnant animals of three species: mice, rabbits, and rats. The mice used were Swiss albinos, Black and A/HC strains. Daily doses of 125-250 mg/kg of thalidomide with food was given from day one to day 14 or day six to day 14 of pregnancy produced 40-50% fetal resorption and five to 11% gross malformations of the survivors. The most frequent abnormalities were: hare lip, cleft palate, cataracts and various tail deformities. In the rabbit, daily doses of 125-250 mg/kg mixed in food from day six to day 14 produced fetal resorptions. Among the surviving fetuses, 30% exhibited malformations of the central nervous system, the limbs, and the axial skeleton. Encephalocele, anencephaly, clubbed feet and deformities of the spinal column associated with leg-defects were described.

In the rats, the three strains investigated by Giroud, Tuchmann-Duplessis and Mercer (1962) were Wistar, Long-Evans and August. Thalidomide was added to the food from day one to 14 or from day six to day 14 of pregnancy in doses ranging from 250-500 mg/kg. This treatment resulted in 20-40% fetal resorption, however, gross malformations were not observed.

Confirmation of the teratogenic effects of thalidomide was demonstrated by Spencer (1962), in the two Himalayan does in which five-tenths of thalidomide was given by stomach tube daily, from day eight to day 14 of pregnancy. The first doe produced a litter of four, one was still-born and all the
litter had marked varus deformities of the hind-limbs. The second doe was killed and the uterus was found to contain a second dead fetus with varus hind-limb deformities and marked foreshortening of the right fore-limb. In both litters the animals which were born alive died within three days.

Since laboratory investigation on the teratogenic properties of thalidomide have been undertaken, many curious and contradicting results have been reported. Hughes, Delhanty, Jay, Chitham, Playfair and Hopper (1962) established tissue cultures from two abnormal fetuses and a minimum of 50 karyotypes per tissue were examined from skin, lung, kidney and testes in primary culture. There was no evidence of chromosome mosaicism in the limited number of tissues studied. The abnormal limb tissue was not studied because at the time it was not realized that phocomelia could be caused by thalidomide.

In an investigation by Luers (1962), thalidomide was administered to drosophila using the standard Müller-5 technique after feedings of five-tenths per cent solution of the substance for three days to imagoes. The results indicated that there was no mutagenic activity of the compound, neither in sperms, nor in earlier stages of spermatogenesis. From these results, it was concluded that male germ cells of drosophila were unaffected.

According to Felisati (1962), thalidomide was given orally as a one per cent suspension in a carboxy-methyl-cellulose solution, 50 mg/kg of body weight daily to Sprague-
Dawley rats and rabbits. In the rats the doses were started the day after coupling and was continued until the twenty-first day. Malformations were not found in the offsprings of the rats. Thalidomide was given to five rabbits seven days after coupling and was continued until the fifteenth day of pregnancy. One of the five rabbits was killed just before delivery, the other two that became pregnant gave birth naturally. Of the 17 fetuses, eight had misshappen anterior limbs. The abnormalities consisted of a folding of the ulna and the radius and hence a shortening of the forearms, and a hook-like forepaw. In some the big toe was missing altogether and in others it was deformed; some haemorrhage was evident over the deformed carpus. Hydrocephalus was found in four fetuses and the middle lobe of the lung was missing in one.

The investigations of King (1962) and Dwornik and Moore (1965) indicated that thalidomide has a definite effect on the ossification of bone, particularly of the sternum and vertebral column. Their work demonstrated that an increase in the dosage of thalidomide was accompanied by an increase in the number of sternabra missing or a delay in the ossification of them. Accordingly, Felisati (1962), noted that the dosage is extremely significant in determining the extent of damage and frequency of the malformation.

Gross malformations were observed in fully formed embryos, both living and dead, as a result of thalidomide.
Injections of doses of 31 to 350 mg/kg body weight (DiPaolo, Gatzek and Pickren, 1964). The most frequent anomaly observed was an open-eye, approximately ten per cent, and the only malformation seen in the control animals was one case of open-eye. In addition, embryos from treated mothers had a variety of abnormalities which varied in severity and laterality, such as enlarged skull on one side, curvature of the back, kinky tails and a few cases of phocomelia and micromelia. In the gross autopsy studies, observation of abnormalities was limited to the bony structures. The vertebral column was frequently elongated and tortuous. The rib cage and limbs were shortened and ribs were missing. In some cases the brain was enlarged. There were no observable gross abnormalities of the thoracic and abdominal organs.

Woollam (1962a), after successfully obtaining malformations in mice, indicated that the most significant single factor that has to be considered by the experimental teratologist is when the noxious agent should be administered. The fetus is sensitive to the effects of such agents only during a limited period in the early part of pregnancy. Moreover, there is for each structure in the body a shorter critical period when it is especially vulnerable. Generally, this period which differs from structure to structure coincides with the time of greatest mitotic activity.
CHAPTER III

MATERIALS AND METHODS

Ninety-six Swiss-Webster albino mice were obtained from the Rockland Farms formerly of New City, New York, presently located at Gilbertsville, Pennsylvania. The mice were eight to nine-weeks old and weighed 25-30 grams.

A five gram sample of thalidomide, α-N-phthalimidoglutarimide, was donated for research purposes by Mr. Leon Lemon. A stock suspension of thalidomide was prepared by mixing 250 mg of thalidomide in 125 cc of solvent, a 30% propylene glycol solution.

The experimental work was undertaken in three phases. In the first phase eight experimental males were given a series of five intratesticular injections of thalidomide at 60 mg/kg of body weight per day. Control males received injections of the suspension medium concurrently with experimental mice through the same route. This phase was designed to produce deformities in progeny fathered by thalidomide injected males. The progeny of thalidomide treated males were designated as Group I.

In the second phase eight experimental females were given a series of five subcutaneous peritoneal injections of thalidomide of 60 mg/kg of body weight per day. Control females were injected concurrently with experimentals by way of the same route. The purpose of this phase was to produce
deformities with thalidomide in the progeny as a result of the mothers having received thalidomide injections prior to conception. These progeny were designated as Group II.

In the third phase six pregnant mice were given a series of five injections of thalidomide at 60 mg/kg of body weight for five days postconception. This phase was designed to produce deformities with thalidomide in the progeny similar to those which occurred in human infants when thalidomide was taken by pregnant women during early stages of pregnancy. The progeny were designated as Group III.

Indication of mating was determined by examination of females at regular intervals for the presence of a copulation plug in the vagina. Discovery of the copulation plug was considered as zero time in the gestation period. In addition to observing vaginas for copulation plugs, vaginal smears were made in order to determine the presence of spermatozoa in the vaginal canal.

A second experiment involving 48 additional mice were undertaken. The strains of mice, ages, weights, dosages and routes were the same as utilized in the first experiment. This experiment was undertaken because mice failed to mate after two months.

Testes and ovaries from injected males and females were prepared for histological studies, according to the paraffin method, and were stained with Harris' hematoxylin and eosin.
CHAPTER IV

EXPERIMENTAL RESULTS

OBSERVATIONS OF THE DEFORMED NEW-BORN MICE

Experimental production of abnormalities in new-borns was successfully accomplished by injections of thalidomide. Gross analysis was made of the new-born mice and the incidence and severity of external malformations was determined. Malformations were manifested in progeny sired by thalidomide injected males, in progeny of thalidomide injected mothers prior to conception and in progeny of thalidomide injected mothers postconception. Macroscopic observations were from 25 litters of the experimental parents and ten litters from the control parents.

There was a marked death rate among all progeny of the experimental parents. Approximately 50% of the young mice were still-born, while those that were born alive and deformed, died shortly after birth or a few hours thereafter.

Thalidomide Induced Malformations

Deformities of the tail.—Gross malformations were observed in tails of new-borns, both living and dead. The malformations were manifested as shortened and kinkiness of the tail. The frequency of this malformation was approximated at five-tenths per cent, in contrast to complete absence of tail deformities in the control mice. Observations
also revealed complete absence of tails in two mice.

Deformities of the back.--The less frequent abnormality observed was curvature of the back. This malformation was observed in youngs which were still-born as well as those which were born alive. The youngs, so afflicted, made no attempt to walk or move from positions in which they were placed.

Deformities of the limbs.--Macroscopic observations of 25 litters revealed 70\% of limb deformities. Normal forward movement was prevented due to malrotation of hind-limbs. This deformity occurred in the hind-limbs only. In addition to malrotation, hind-limbs were shorter than those of the controls. Shortening of the hind-limbs occurred more frequently than shortened fore-limbs. In many cases limbs were shortened only on one side of the body. This applied to both fore- and hind-limbs. Shortened hind-limbs, in most cases, were accompanied by twisting of limbs toward the body or away from it, thereby making normal movement impossible.

Gross analyses of limbs revealed an absence of some of the toes and syndactyly in some mice, while in others, club-footedness was observed. A striking malformation was the attachment of a hind-limb to the abdominal region, an obvious case of phocomelia. In association with this abnormal attachment, these limbs were collapsed and tortuous. Another outstanding anomaly was the attachment of accessory structures to already deformed limbs.
Deformities of Pelvic and Head Regions.—The most outstanding malformation observed in the 25 litters was an absence of the pelvic region. Although this was a prominent malformation, it was observed in only two mice which succumbed a few minutes after birth. In two other mice of the same litter the extremities of the pelvic region were flaccid and extremely tortuous. There were no such deformities observed in the control mice.

In 25% of all young mice deformities of the head region were observed. The most prevalent deformity in this region was absences or malformations of the mouths. Mice having mouth deformities were unable to suck and consequently were short-lived. Associated with mouth deformities were deformities of the ear lobe. In the same young mouse there was a complete absence of the pinna while in others only a rudimentary structure was present.

Description of the Control Testis

The testis is an ovoid compound tubular gland surrounded by a fibrous membrane, the albuginea testis. The interior is filled with extremely tortuous convoluted seminiferous tubules in which the sperms are formed. A section through the adult mouse testis (fig. 1) shows the seminiferous tubules with clusters of interstitial cells compactly scattered in the angular spaces between the tubules. The interstitial or Leydig cells are responsible for manufacturing the male
hormone which stimulates the production of sperms in the tubules.

In a mature male mouse the seminiferous tubules are lined by two kinds of cells, the cells of Sertoli which are nutrient, supporting cells and the sex or spermatogenic cells from which sperms develop through the process of proliferation and transformation. A basement membrane surrounds the tubules to which the cells of Sertoli and spermatogonia are attached. Separating the Sertoli cells at regular intervals are the spermatogenic cells.

The cells at the onset of spermatogenesis are the spermatogonia. These cells undergo a period of growth during which they increase in size and the nuclear content becomes more distinct. While in this growth phase the cells are gradually shifted toward the lumen of the tubules. The cells at the termination of the period of growth are called primary spermatocytes. These cells enter a period of maturation during which the primary spermatocytes divide into two cells, the secondary spermatocytes. These cells exist only for a short time and then divide into two cells the spermatids. The spermatids represent the completion of maturation and are transformed into spermatozoa. The heads change into long spear-shaped bodies while the tails become slender and elongated. The sperms then leave the wall of the tubule and pass into the lumen from which they propel themselves to the vas deferens.
Description of the Thalidomide Injected Testis

Beneath the fibrous albuginea testis many of the tubules appeared loosely suspended due to the absence of interstitial cells. Only fragments of these cells were observed between the tubules (fig. 2). In addition to the tubules being loosely suspended, dissolution or partial dissolution of the basement membrane of many of the tubules was observed (fig. 3). Only viscous appearing strands were present.

In tubules where the basement membrane persisted, large irregularly shaped cells were loosely attached and had the appearance of spermatogonia (fig. 4). The second and third tiers of cells, the primary spermatocytes, are smaller and more irregularly shaped. The cells nearest the lumen are diffused and exhibited a variety of shapes, from globose to stellate-like. In the same tubules, the lumen was occluded with a granular-like debris (fig. 5), but was completely void of sperms (fig. 6).

The tubules in which partial dissolution of the basement membrane was evident, the cells appeared loosely suspended with little or no intercellular cement between them (fig. 7). These diffused cells were of two definite cell types, spermatogonia and primary spermatocytes (fig. 8).

In other tubules in which complete dissolution appeared to have occurred, the cells that lined the tubule, both Sertoli and spermatogonia appeared to have been
dissolved (fig. 9). Only the cells near and in the lumen were distinct and did not have the appearance of maturing germ cells. In no instance were sperms found in the tubules.

In tubules in which the basement appeared unaffected, only matrix-like material was observed (fig. 9). Diffused large cells filled the remainder of the tubule. In addition to these large cells which filled the lumen of the tubules, a section through the epididymus revealed the presence of the same cell shapes and varied sized cells instead of sperms as in the control testis.

In the lumen of tubules, in which maturation was complete, sperms of various shapes, globose, ellipsoid and stellate were found. Associated with these diverse shapes of sperms a fibrous-like debris was present (fig. 10).

Tubules in which both basement membrane and intercellular cement had been retained, the cells were only three to four tiers thick, which left a large lumen void of any cells.
CHAPTER V

DISCUSSION

The results obtained in this investigation clearly indicate that injections of thalidomide cause abnormalities of varying degrees in Swiss-Webster albino mice. Although deformities of the limb were most frequent, deformities of the mouth, ears, tails and pelvic regions were also observed.

Studies on embryonic development and teratology have revealed that there are definite and well defined stages of embryonic development during which teratogens and noxious agents will change the fate of particular parts. According to Woollam (1962 a), the fetus is sensitive to thalidomide only during the "critical period" of parturition. Furthermore, the susceptible periods vary from organ to organ. They frequently coincide with the first visible appearance of a particular primordium, however, the sensitive period may precede the time at which a primordium can first be recognized as a morphological entity (Landauer, 1953).

Woollam stated that the incidence of malformation of particular structures vary with the amount of dosage and time of administration. In another investigation by Woollam (1962 b) he observed that when the dosage was doubled, difficulties in parturition were experienced and the frequency of malformations increased.

The greatest number of abnormalities were observed in new-born mice whose mothers received injections of thalidomide
prior to pregnancy. This fact tends to indicate that a sufficient accumulation of thalidomide was acquired before the critical period was reached and the deleterious effect of this noxious agent was manifested by a high frequency of deformities and a marked death rate.

The high death rate may be explained on the basis that the dosage of the teratogen might have reached the toxic level before the level of teratogenesis was accomplished. Accordingly, Cahen (1961) stated that many drugs which are toxic do not produce malformations regardless of the amount of the dosage. This fact would account for many of the mice in this investigation being still-born.

Since abnormalities of the limbs were more frequent in the hind-limbs than in the fore-limbs, it appears that the thalidomide exerted its effect during the susceptibility stage of these structures. DiPaola (1963) proposed a possible explanation of this occurrence by stating that the hind-limbs are slower to appear than the fore-limbs, thereby, rendering them more susceptible to thalidomide.

According to Landauer (1953), micromelia is presumably the result of a disturbance in the growth of long bones. It seems likely that the abnormalities of the appendicular skeleton are likewise the result of a failure of the constituent cartilaginous primordia to grow normally. In particular circumstances, disharmonies and dislocations of growth result in gross malformations such as syndactylism and bending of the limbs and tail.
The experimental production of abnormalities appears to depend directly on the amount of the dosage and the time at which thalidomide is administered. The production of deformities in progeny of thalidomide injected males is an indication that males as well as females can risk deformities in their offspring after taking dosages of the teratogen. Observation of the testes of these thalidomide injected males revealed some interesting results.

This phase of the investigation involving observations of thalidomide injected testes clearly revealed a deleterious effect on spermatogenesis. Partial or complete dissolution of the basement membrane of the tubules may have been due to thalidomide which may have had an affinity for its lipoprotein components, thereby causing a breakdown of the membrane. In the tubules in which the basement membrane persisted the large irregularly shaped cells appeared to have been spermatogonia. The presence of these cells throughout the tubule may be a possible indication that the process of spermatogenesis had not proceeded beyond the level of the spermatogonium. Therefore it may be that thalidomide caused developmental arrest of the sex cells.

The absence of intercellular cement may have been due to the action of thalidomide upon the constituent, hyaluronic acid, or it may have caused a hypersecretion of the enzyme hyaluronidase, thus causing an imbalance between enzyme and substrate. If such an abnormal situation occurred the
hyaluronidase would possibly have digested away the intercellular matrix between the cells, which left them free as was observed. It may also be that thalidomide or one of its metabolites may have acted as a solvent for the hyaluronic acid of the intercellular matrix and of the lipo-proteins of the basement membrane of the tubule.

Destruction of the Sertoli cells may also account for developmental arrest and malformation of sperms, since they are nutritive and supporting in nature. The absence of interstitial or Leydig cells would account for the failure of conception to have occurred in the mice during a two-month period after administration of thalidomide. The cells of Leydig are believed to produce the male hormone which stimulate the production of sperms. In the absence of this hormonal influence the sperms could not have formed. The presence of a small number of cells of Leydig may not have produced enough androgen so that the spermatids could be transformed into normal sperms.
CHAPTER VI

SUMMARY AND CONCLUSIONS

The reaction of the developing organism to thalidomide depends upon the following factors:

1. the stage at which it is subjected to thalidomide,
2. the nature of this teratogenic substance,
3. the length of time it is exposed, and
4. the physiological condition of the developing organism.

Thalidomide affects the formation of the sperms by:

1. destroying the cells of Leydig, thereby preventing the formation of sperms,
2. causing developmental arrest of spermatogonia,
3. destroying the intercellular matrix and
4. destroying the cells of Sertoli.
The figures are photomicrographs
(Explanation of Figures)

Fig. 1. Transverse section through a control testis showing tubules (t) with interstitial cells (ic). x970.

Fig. 2. Transverse section through the seminiferous tubule of a thalidomide injected testis showing dissolution and destruction of the basement membrane (bm) x970.
*PLATE II

*The figures are photomicrographs
(Explanation of Figures)

Fig. 3. Transverse section through a tubule of a thalidomide injected testis showing large irregularly shaped (isc) cells loosely attached to the basement membrane (bm). x970.

Fig. 4. Transverse section through a tubule of a thalidomide injected testis showing loosely arranged cells of various shapes. x970.
The figures are photomicrographs
Fig. 5. Transverse section through a tubule of a thalidomide injected testis showing lumen containing matrix-like debris (mld). x970.

Fig. 6. Transverse section through a tubule of a thalidomide injected testis showing dissolution of the basement membrane (bm) and intercellular cement (ic). x970.
PLATE IV

The figures are photomicrographs.
(Explanation of Figures)

Fig. 7. Transverse section through a tubule of a thalidomide injected testis showing partial dissolution of the basement membrane (bm) and breakdown of intercellular cement (ic).   x970.

Fig. 8. Transverse section through a tubule of a thalidomide injected testis showing spermatogonia (s) along the basement membrane (bm) and primary spermatocytes (psp) loosely diffused near lumen (l).   x970.
(Explanation of Figures)

Fig. 9. Transverse section through a tubule of a thalidomide injected testis showing dissolution of both spermatogonia (s) and cells of Sertoli (cS). Large irregularly shaped cells fill the lumen (isc). x970.

Fig. 10. Transverse section through a tubule of a thalidomide injected testis in which maturation appeared to be complete. Sperms of various shapes are located near the lumen (s). A fibrous-like debris fill the lumen (de). x970.
The figures are photomicrographs.


