Effect of Vitamin A on the Potentiality of Rat Palatal Processes to Fuse in vivo and in vitro

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Introduction

The administration of excess vitamin A to pregnant laboratory animals has been used extensively to produce a high incidence of cleft palate in offspring (*8, 9, 11, 24, 25*). However, explanations differ as to how maternal hypervitaminosis A leads to fetal cleft palate. Woollam and Millen (*24, 25*) studied the mechanism of action of vitamin A by combining it with hormones and hormone antagonists and postulated an effect via an alteration of the maternal carbohydrate metabolism. Giroud et al. (*10*) believed that hypervitaminosis A has a direct effect on the fetuses, as they found an increase of vitamin A in the fetal content after its administration to pregnant rats.

Others have reported structural changes in the craniofacial complex of fetuses from vitamin A treated rats which might directly or indirectly influence fusion of the palatal processes. These structural changes include: a short mandible and maxilla (*2*), precocious cartilage formation in the maxillo-mandibular area (*18, 23*) decreased cell proliferation (*12, 19*) and alterations in the metabolism of the palatal processes as evidenced by their increased uptake of S^{35} sulfate (*13*).

Recently, in vitro techniques have provided a means of studying morphological and cellular events that take place in palatal fusion in the absence of surrounding cranio-facial structures, maternal metabolism and placental transfer. Based on these advantages of the in vitro technique, the following problems were studied in the present investigation.

a. It has been reported that in the fetuses of vitamin A treated rats the palatal processes are stunted (*17*) and the lateral growth of the head continues "normally" or unhindered, resulting in an increased gap between the two palatal processes. Similarly, views have been expressed concerning the effect of other cranio-facial structures on palatal fusion. An in vitro experiment might clarify the question of whether or not the palatal processes from fetuses of vitamin A treated rats, obtained on

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different days of gestation, still have the potential to fuse in the absence of maternal metabolism and environment.

b. It has been suggested that hypervitaminosis A causes cell shrinkage with fluid accumulation in the intercellular spaces (*14*) and reduction in cell proliferation and mitotic rate (*12, 14, 17*). An in vitro experiment could show whether vitamin A added to the culture medium has a direct effect on the potential of normal palatal processes to fuse with each other.

The present work used in vivo experimentally induced clefts to study the morphogenesis of the malformation. In addition, in vitro techniques were used to study the ability of palatal processes of fetuses from vitamin A treated rats to fuse in the absence of maternal influences and the ability of palatal processes from normal fetuses to fuse in the presence of vitamin A added to the medium.

Materials and Methods

Female Wistar albino rats were placed overnight with the male rats of the same strain. The animals were checked in the morning for the presence of vaginal plug. If found positive, day 0 of pregnancy was recorded. The pregnant rats were divided into three groups for experimental purposes.

Group I. Fifteen pregnant rats were used in this group as controls. The rats were sacrificed by cervical decapitation at 15, 16, and 17 days of gestation. The 15 and 16 day old fetuses were aseptically removed and were placed in a dissecting dish containing Tyrode's solution and horse serum (4:1, v/v). To obtain palatal explants, a transverse incision was made through the oral opening of the fetus and the head portion was separated from the mandible and the rest of the body. The maxillary portion was resected by making an incision parallel to the previous one at 1 to 1.5mm more cranially. The tissue lying posterior to the palatal processes was cut and discarded. If still present, the tongue was removed. The dissected palatal explant included the primary palate, the maxillary ridges, the palatal processes, and part of the nasal septum (Figure 1).

The basic medium used in the present study contained NCTC 109 with 10% calf serum and 50 u/ml penicillin. Immediately after dissection, explants were placed with the nasal surface down on an Organ Culture Grid (Baltimore Biological Laboratory, Cockeysville, Maryland). Explants were cultured in an atmosphere of 5% CO_2 and 95% air. After the termination of the incubation period the cultured explants were fixed in Bouin's solution, embedded in paraffin, and 7 μm sections were cut serially. The sections were stained with haematoxylin-eosin. In this group a total of twelve pregnant rats were used for in vitro experiments and a total of 35 explants were obtained for culturing. Two fetuses from each mother were fixed in Bouin's solution, embedded in paraffin and 7 μm sections were utilized for histological examination.

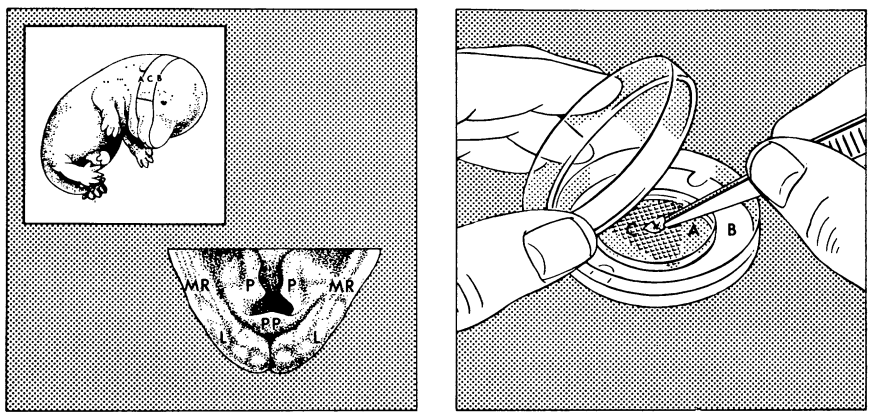

FIGURE 1. Diagrammatic representation of the cutting procedure employed for preparing explants. In the left upper figure (A) denotes the first cut separating the head from the mandibular region and the rest of the body, (B) is the second cut, cranial to the previous one, and (C) is the part discarded and the anterior section was used for culturing. The left lower figure is the oral view of the part used for culturing includes primary palate (PP), palatal processes (P), maxillary ridges (MR), and the labial area (L). The right diagram shows the organ culture dish with the palatal explant on a stainless steel grid (C). One ml. of culture medium was placed in the center well (A) and 5 ml. of water was poured on the absorbent ring (B).

The remaining three rats were sacrificed at 17 days of gestation. The fetuses were utilized for histological examination. The sections were stained with haematoxylin-eosin.

GROUP II. This group consisted of 30 pregnant rats. The rats were given daily oral doses of 40,000 I.U. vitamin A palmitate from day 9 to 13 of gestation.

Eight rats were sacrificed on the 15th day and 7 rats on the 16th day of pregnancy for in vitro experiments. The dissection, culture and histological preparation methods were exactly the same as described for Group I. A total of 30 explants were incubated for 3 to 8 days. Every three days the explants were transferred to a fresh culture medium, without disturbing them on the grid.

The remaining 15 pregnant rats were sacrificed at 17 (8 rats) and 18 (7 rats) days of gestation. The fetuses were studied macroscopically and histologically. The histological technique was similar to Group I.

GROUP III. In this group 30 palatal explants were obtained from twelve pregnant rats at 16 days of gestation. The technique of dissection, culture, and histological preparation was the same as described for Group I, except that vitamin A palmitate was added in the culture medium. The 30 explants were divided into three equal groups, and each group received either 10, 25, or 50 I.U. vitamin A per ml. in the medium. Incubation period ranged from 3 to 8 days. The harvested explants were prepared for histological examination in a similar manner as described for Group I.

When the explants were cultured for an extended period, the culture medium was replaced every 3 days with appropriate concentrations of vitamin A added.

Results

The morphological and histological examination of fetuses obtained from untreated rats of Group I revealed no abnormality. The 17 day old fetuses showed complete fusion of palatal processes (Figure 2).

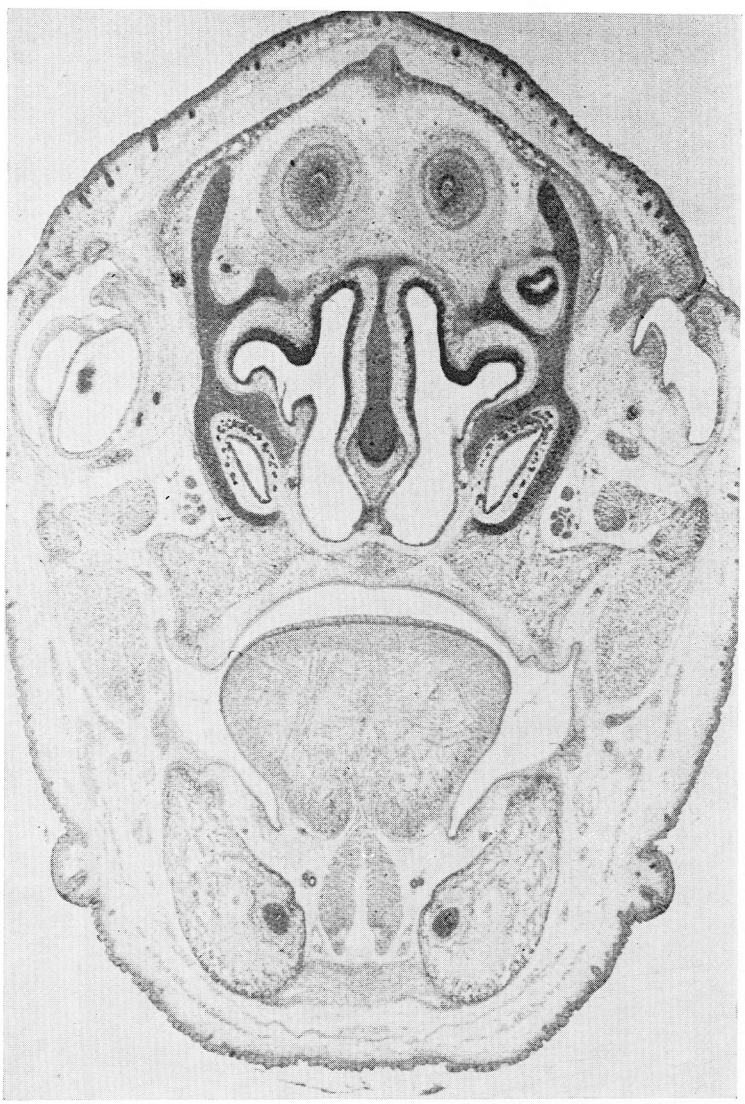

FIGURE 2. Normal rat embryo, frontal view. The secondary palate is fused. H&E × 15.

Excessive doses of vitamin A to pregnant rats caused cleft palate in 86% of the living fetuses when examined on days 17 and 18. Histological and macroscopic examination revealed several other malformations such as microstomia, retrognathia, maxillo-mandibular ankylosis, short and bulbous nasal septum, and tongue lying between the malpositioned and malformed palatal processes (Figure 3).

The cultured explants were examined for fusion or non-fusion of the opposing palatal processes by studying serial sections histologically. The palatal processes were divided into three categories based on histological examination: 1. no fusion—when two opposing palatal processes did not come in contact with each other; 2. epithelial fusion—limited adherence with a short epithelial bridge or only epithelial contact; and 3. mesenchymal fusion—when epithelial contact was broken at one or more areas.

Out of 35 control palatal explants obtained from untreated rats (Group II) and incubated for three days, ten showed some form of epithelial fusion, 21 showed mesenchymal fusion, and four showed no fusion (Table 1). Even the four non-fused explants showed appreciable increase in the

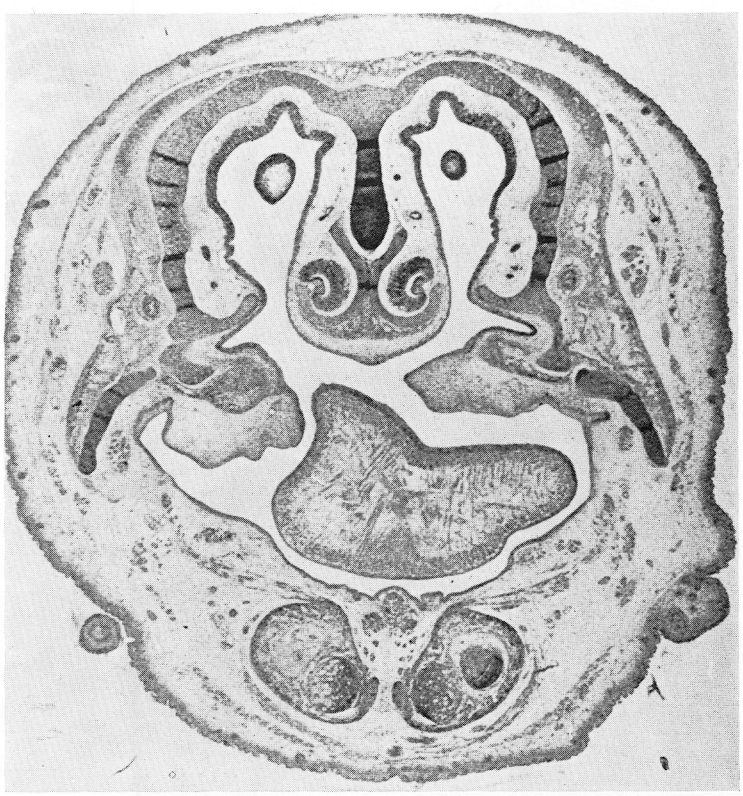

FIGURE 3. Rat embryo from vitamin A treated mother. The palatal processes are unfused and deformed. The tongue is protruding on one side into the nasal cavity. Aberrant cartilage can be seen in the area lateral to the palatal processes. H&E × 15.

TABLE 1. Incubation time, number of explants, and status of fusion or non-fusion of palatal processes.

age of the explant at the time of culture (days)	*no. of explants*	*incubation time (days)*	*epithelial fusion*	*mesenchymal fusion*	*no fusion*
Group I*					
15	16	3	6	8	2
16	19	3	4	13	2
Group II**					
15	4	3	0	0	4
	4	1	0	0	4
	3	6	2	0	1
	3	8	3	0	0
16	3	3	1	0	2
	4	4	3	0	1
	4	6	2	2	0
	5	8	1	2	2

* Normal explants cultured in normal medium.

** Explants obtained from fetuses of vitamin A administered rats and cultured in normal medium.

size of the palatal processes. Mesenchymal fusion was seen more often in 16 day old explants as compared to 15 days (Figure 4).

The palatal explants obtained from the fetuses of vitamin A treated mothers and cultured in normal medium (Group II) exhibited no fusion until the third day of incubation (Table 1). An extended incubation period resulted in a number of fused palatal processes. In total, out of 30 palatal explants, sixteen showed some degree of either epithelial or mesenchymal fusion. Mesenchymal fusion was observed in only four explants which were from 16 day old fetuses incubated for six days (Figure 5).

When normal explants were cultured in the presence of vitamin A (Group III) only at the lowest vitamin A concentration (10 I.U./ml) did two explants out of ten show epithelial fusion (Table 2). The explants grown in a medium containing 25 or 50 I.U. of vitamin A per ml of medium showed no form of fusion of the palatal processes. The epithelium of the palatal processes was found to broken or peeled off from the mesenchyme at the oral surface at several areas. The epithelium facing the nasal chamber appeared intact but was edematous. In several explants, the epithelial layer showed cornification. Overall, in cultures where vitamin A was added, the palatal processes appeared small and less developed as the gap between the two opposing shelves was greater as compared to explants from Group I and II (Figure 6).

Discussion

The findings that palatal fusion in the untreated fetus occurred at the 17th day of gestation and that maternal hypervitaminosis-A yielded clefts

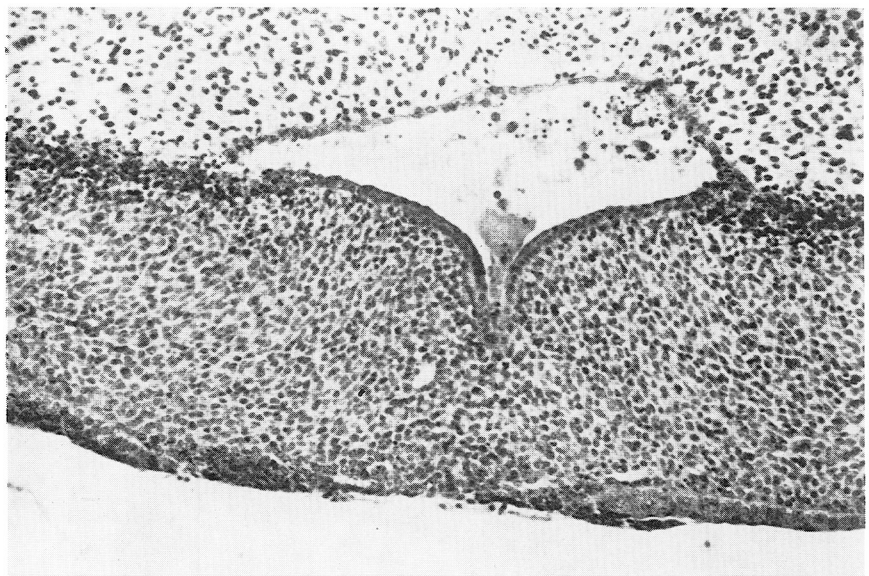

FIGURE 4. Normal explant cultured in the normal medium for 3 days, showing mesenchymal fusion. H&E × 150.

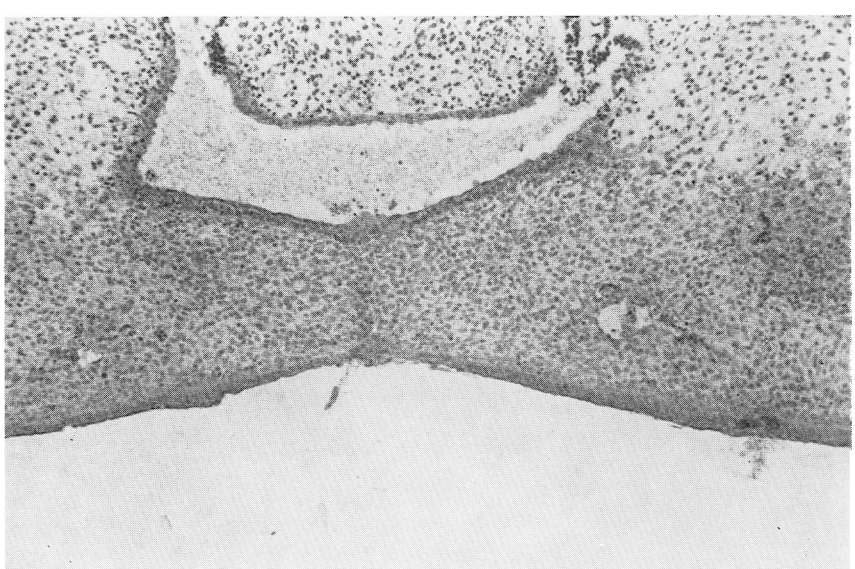

FIGURE 5. Explant from a 15 day old embryo of vitamin A treated mother and cultured on normal medium for 8 days. Note complete mesenchymal fusion of the palatal processes. H&E × 150.

TABLE 2. Palatal processes dissected on day 16 of gestation and cultured in a vitamin A supplemented medium.

concentration of vitamin A (i.u./ml of medium)	*no. of explants*	*incubation time (days)*	*epithelial fusion*	*mesenchymal fusion*	*no fusion*
10	2	3	0	0	2
	4	6	1	0	3
	4	8	1	0	3
25	2	3	0	0	2
	4	6	0	0	4
	4	8	0	0	4
50	2	3	0	0	2
	3	6	0	0	3
	5	8	0	0	5

in 86% of the viable fetuses are consistent with observations previously reported in the literature (*2, 3, 8, 13*).

The notable observations of the present study were from Group II, where the palatal explants from vitamin A treated mothers were cultured on normal medium. Sixteen explants exhibited some degree of epithelial or mesenchymal fusion after a prolonged period of incubation. This finding

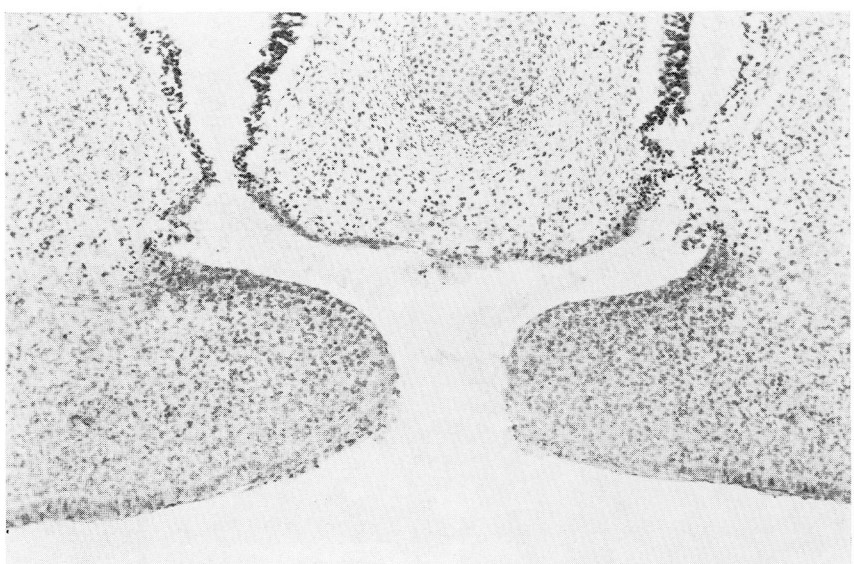

FIGURE 6. Explant from a normal embryo cultured on 10 I.U./ml vitamin A added medium for 8 days. Note the palatal processes are unfused. The palatal epithelium is under degeneration. H&E × 150.

disagrees with the in vivo findings of the present study and views expressed in literature; however, no parallel experiment has been performed. It has been reported that hypervitaminosis A causes cleft palate in laboratory animals by disturbing maternal carbohydrate metabolism (*24, 25*), by alterations in the mucopolysaccharide metabolism (*13*), and by decreased cell proliferation of the palatal processes (*12*). These authors did not mention whether the potential of the palatal processes to fuse with each other is completely lost or not. The present findings reveal that palatal processes from vitamin A treated mothers had the potential to fuse in vitro although only after prolonged incubation. These results lend "indirect" support to the hypothesis that growth of the head in width in vivo and changes in other structures such as non-lowering of tongue and micrognathia play an important part in increasing the gap between the two palatal processes. The initial gap might be due to decreased cell proliferation or arrested growth of the palatal processes as has been shown by Kochhar (*12*) and Nanda (*19*).

The second hypothesis which was tested in the present study was whether palatal processes from untreated mothers had the potency to fuse in the direct presence of vitamin A. The results support the findings of Myers et al. (*16*) and Pourtois (*21*) that vitamin A in direct contact with palatal shelves in vitro retards their growth and fusion does not take place.

The particular observation in the present study regarding cornification of the palatal epithelium after 6 to 8 days of culturing palatal processes in vitamin A added medium (Group III) supports the findings of other workers (*5, 6, 14*) regarding the effect of vitamin A. However, these studies have only shown the effect of hypervitaminosis A on embryonic bones and skin. It is probable that in palatal explants where the palatal processes came in contact with each other and did not fuse, cornification of the epithelium might have played an important role in the loss of their ability to fuse with each other.

In Group III only 16 day old palatal explants were cultured, as it was evident from Groups I and II that the incidence of palatal fusion with 16 day old explants was higher than at 15 days. This difference can be explained by the presence of a narrow gap between the two opposing palatal processes of 16 day old explants as compared to the 15 day old cultures. Viable comparisons regarding this finding cannot be drawn with Pourtois' study (*21*) on the potentiality of the palatal processes to fuse in vitro. He placed completely detached palatal processes in close proximity with each other on the culture medium, and thus the palatal processes missed the stage of growing towards each other; whereas, in the present study, a fusion of the palatal processes involved growth of palatal processes towards each other and subsequent fusion of the two processes.

The present study suggests that vitamin A retards the growth of the

palatal process in vivo and subsequently the processes do not come in contact with each other at the morphogenetically determined time. The growth of the head subsequently moves the processes apart and fusion does not take place. However, the palatal processes retain their potential to fuse in vitro in the absence of cranio-facial structures and maternal metabolism and environment. This further suggests that vitamin A probably does not irreversibly disturb the normal in vivo events of fusion mechanism which have been reported by Angelici and Pourtois (*1, 21*), Farbman (*4*), and Smiley and Dixon (*22*) in recent electron microscope studies. However, the possibility remains that vitamin A may inhibit a biochemical event crucial to fusion of the processes in vivo and during prolonged culture in vitamin A free medium the processes are released from this inhibition due to the absence of maternal environment. Gal et al. (*7*) recently reported their findings on the relation of vitamin A to human congenital malformations. They found a relatively high vitamin A concentration in maternal blood and fetal liver of malformed fetuses, suggesting a possible teratological effect of excess vitamin A in humans. The findings of the present study and Gal et al. (*7*) need further elaboration by experiments studying the distribution and metabolism of excess vitamin A and its role in cleft palate production and other congenital malformations. Further experiments are in progress on this subject.

Summary

The effect of hypervitaminosis A on the potentiality of the rat fetal palatal processes to fuse in vivo and in vitro was studied by utilizing three experimental models. In the first, excessive doses of vitamin A were given to pregnant rats; in the second, excessive doses of vitamin A were given to pregnant rats and palatal explants of fetuses were grown in vitro; in the third, normal palatal explants were cultivated on vitamin A added medium. Specimens were studied macroscopically and histologically. The findings reveal that palatal processes obtained from fetuses of vitamin A treated mothers retain the potentiality to fuse in vitro in the absence of maternal metabolism and environment.

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