The effect of hydrocortisone acetate on the development of mouse embryos

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Hydrocortisone (Kendall's compound F, 17-hydroxycorticosterone, Cortisol) and cortisone (Kendall's compound E, 17-hydroxy-11-dehydrocorticosterone) have been widely used in medicine for about 20 years, first as adrenal hormones in substitution therapy. Since the discovery of Hench, Kendal, Slocumb & Polley (1949) that cortisone is beneficial against a wide range of rheumatic diseases and against all forms of arthritis, many more types of diseases have been added in later years to the list of ailments curable with cortisone, namely bronchitis, bronchial asthma, pulmonary tuberculosis, colitis, various inflammatory conditions and certain skin, blood and eye diseases. Hydrocortisone is used against the same diseases as cortisone; in fact, it is believed that cortisone becomes pharmacologically active only after conversion to hydrocortisone in the liver (Cope, 1964). Particularly in the inflammatory conditions cortisone must be converted to hydrocortisone to become antiphlogistic (Applezweig, 1962).

The biochemical mode of action of these drugs was not understood for many years. More recently, hydrocortisone in particular has been used extensively in investigations in the field of cell biology, particularly in investigations of protein synthesis. In general, it is believed that at the cellular level hydrocortisone influences many metabolic processes. First, it has a cell-stabilizing effect which can be expressed for instance in its antihemolytic activity. It stabilizes lipoprotein membranes in general, and in particular prevents lysosomes from releasing the hydrolytic enzymes contained inside these organelles (de Duve, Wattiaux & Wibo, 1961). The latter effect may explain the mechanism of the protective properties of hydrocortisone against cell damage induced by such factors as irradiation with ultraviolet light or X-rays or intoxication with bacterial toxins or chemical agents (Weissmann & Dingle, 1961; Weissmann & Fell, 1962; Weissmann & Thomas, 1963).

However, hydrocortisone does not always protect cells from injury or alleviate damage. On the contrary, in some cases it delays the repair of cellular injury. This paradoxical phenomenon is most probably due to occasional stimulation of the synthesis of enzymes deaminating amino acids, which is followed by delayed protein synthesis (Kenney, 1962a, b; Segal & Kim, 1963; Schimke,

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Sweeney & Berlin, 1964). In other cases stimulation of the synthesis of other enzymes may be more constructive, e.g. it may enhance the formation of glycogen by activating the enzymes responsible for glycogenesis (Jacobson, 1964).

It was found, moreover, that the induction of enzyme synthesis is brought about following an increase in nuclear RNA synthesis (Kenney, Greenman, Wicks & Albritton, 1965). This was confirmed by Sekeris & Lang (1964), who were able to show that in the rat liver messenger RNA synthesis is increased. The increase in DNA-dependent RNA synthesis, and subsequently in protein synthesis, after administration of cortisone and hydrocortisone modifies the intracellular enzymic environment. The newly synthesized enzymes may then impair the later synthesis of specific proteins that are essential for growth and differentiation in developing systems. I would suggest that this is one mechanism by which these hormones cause foetal mortality, stunting and decrease in the viability of neonates.

In medicine, the effects of cortisone are not too disappointing. For example, Bongiovanni & McPadden (1960), summarizing the clinical literature, cite that about 10% of 260 human pregnancies resulted in still-born and premature infants after cortisone medication at various stages of pregnancy.

As far as animal experimentation is concerned, except for investigations on the influence of cortisone and hydrocortisone on the growth rate of chick embryos (Sames & Leatham, 1951) and on the uranoschitic activity of these hormones, there has been only one report of malformations caused by cortisone (Moscona & Karnofsky, 1960). Hydrocortisone has not been investigated in this respect.

This paper attempts to deal with the embryopathogenic properties of hydrocortisone acetate, mainly with regard to limb development in mice.

MATERIAL AND METHODS

The pregnant mice used in the present investigations were of the JBT strain inbred by brother–sister litter-mate matings for twenty-four generations with the retention of sublines. This strain was derived from the outbred JC strain selected for genes $a$, $b$ and $bt$. The day on which the copulation plug was found was considered the first day of pregnancy.

The hydrocortisone acetate used was obtained in pure crystalline form from Calbiochem, Los Angeles, or in the form of Hydrocortistab Boots (hydrocortisone acetate injection B.P.) for intra- and peri-articular injections. In each case the drug was prepared as a suspension in 0.9% saline solution and was injected subcutaneously under slight ether anaesthesia, usually in three doses on the 10th, 11th and 12th days of pregnancy. The dose applied was estimated to be just below the ‘embryonic LD50’ for the hormone. The ‘embryonic LD50’ for hydrocortisone acetate is defined as the total dose which, if administered subcutaneously on the 10th, 11th and 12th days of pregnancy kills about 50% of
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the implanted embryos by the 14th day of pregnancy. The ‘embryonic LD50’ of hydrocortisone acetate is 84 mg/kg (i.e. 28 mg/kg on each of the three injection days). The surviving embryos show the abnormalities to be described. To increase the proportion of the surviving embryos a slightly lower dose of 25 mg/kg was injected.

The animals were sacrificed by cervical dislocation on the 14th day of pregnancy (291 pregnant females, 1382 surviving embryos) or on the 18th day of pregnancy (104 pregnant females, 407 surviving embryos). Dissected uteri were fixed in toto in 5% trichloroacetic acid containing 1-37% lanthanum acetate, and the embryos were dissected at least 3 h later. Some embryos after immediate dissection from the uterus were fixed with 3% glutaraldehyde buffered at pH 7-2 with cacodylate buffer (Plumel, 1948).

The embryos were first examined macroscopically and any gross abnormalities recorded. Selected whole embryos or isolated limb buds were prepared for histological examination in the usual way and stained with methyl green-pyronine. This staining method works particularly well after 3% glutaraldehyde fixation.

In order to determine the possible influence of hydrocortisone on growth rate, the experimental and control embryos were fixed on the 14th day of pregnancy, dissected, weighed, and the averages compared.

Limb buds showing necrotic changes and haemorrhages as well as those with necrotic areas in the innermost parts of cartilage condensations were also prepared for electron microscope examination. For this purpose the 14-day-old embryos were dissected and fixed in 1% osmic acid solution buffered with veronal-acetate buffer at pH 7-2 (Palade, 1952) in Caulfield’s modification (1957) for 45 min at 2–4 °C. During the dehydration procedure the limb buds were dissected and embedded in Araldite, using a slow rotary shaker (Jurand & Ireland, 1965).

Control material was collected in the same manner from 152 untreated pregnant females which were only slightly etherized on the days corresponding to the injection days. In addition, all relevant information contained in a previous paper (Jurand, 1965) was used for comparison.

RESULTS

Macroscopical observations

Fourteen-day-old embryos. 150 experimental and 163 control 14-day-old embryos were used for testing the influence of hydrocortisone on the growth rate. There was no significant difference in body weight between the experimental and control embryos:

<table>
<thead>
<tr>
<th></th>
<th>Number of embryos</th>
<th>Average weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control embryos</td>
<td>163</td>
<td>0-103 g</td>
</tr>
<tr>
<td>Experimental</td>
<td>150</td>
<td>0-105 g</td>
</tr>
</tbody>
</table>
Macroscopically, 26% of the surviving 14-day-old experimental embryos showed one or more abnormalities. For convenience, the effects are classified into three groups according to the degree of damage and to the presumed order of events.

Primary effects are those which include macroscopically visible dilatation of the venous marginal blood sinus (marginal vein) of any of the foot plates without any other macroscopically visible abnormalities (Plate 1, figs. A and B).

Secondary effects comprise macroscopically visible necrotic areas in the limb-bud mesoblast, usually localized in the marginal area of the foot plate (Plate 1, fig. C) or extensive haemorrhages present in the same marginal areas of the limb buds (Plate 1, fig. D). These two types of damage can be present in one or more limb buds without any obvious preference for localization in the fore- or hind-limb buds. To this group of effects also belong changes similar to those in the limb buds but located in the tail tip (Plate 1, fig. E), necrosis of the maxillary processes (Plate 1, fig. F), facial fissures (Plate 1, fig. G) and subepidermal blisters located usually on the temporal region of the head (Plate 2, fig. A) or, less frequently, on the back, limbs or other regions of the body.

Malformations of limbs without any macroscopically visible necrotic changes or haemorrhages resulting in an abnormal outline of foot plates are regarded as tertiary effects (Plate 1, figs. C, E; Plate 2, fig. A). Such deformations are regarded as regulatory effects of healing after previous localized necrotic injuries.

Numerical data on macroscopically detectable effects are shown in Table 1.

To the tertiary effect group belong also histologically detectable, sharply demarcated necrotic changes of the innermost areas of the limb skeletal condensations (Plate 2, figs. E, F). Such changes very frequently accompany the secondary effects, i.e. necrotic injuries and haemorrhages located in the distal parts of the limb mesoblast, but they are often found also in macroscopically quite normal-looking embryos. It is worth noting that in embryos where the

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**Plate 1**

*Fig. A. Control 14-day-old mouse embryo hind-limb bud. ×16.*

*Fig. B. Dilation of the marginal venous sinus in a 14-day-old mouse embryo hind-limb bud after treatment with hydrocortisone acetate. ×16.*

*Fig. C. Experimental 14-day-old mouse embryo with an extensive necrosis of the marginal region in the left forelimb bud. Note also the malformation of the hind-foot plate. ×14.*

*Fig. D. Experimental 14-day-old mouse embryo with an extensive haemorrhage in the distal part of the forelimb bud. ×14.*

*Fig. E. Tail-tip haemorrhage and distinct malformation of the right hind-foot plate in an experimental 14-day-old mouse embryo. ×12.*

*Fig. F. Bilateral extensive necrotic blisters of the maxillary processes resulting in a wide hare lip: 14-day-old mouse embryo. ×10.*

*Fig. G. Bilateral facial clefts. ×10.*
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skeletal condensations show necrotic changes, the non-limb cartilage condensations are not affected by necrosis.

Table 1. Macroscopically detectable changes in 14-day-old embryos
(total, 1382 embryos)

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of embryos*</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilatation of the venous blood sinus</td>
<td>201</td>
<td>14-5</td>
</tr>
<tr>
<td>Distal limb bud mesoblast necrosis</td>
<td>110</td>
<td>7-9</td>
</tr>
<tr>
<td>Distal limb bud haemorrhages</td>
<td>149</td>
<td>10-7</td>
</tr>
<tr>
<td>Tail-tip haemorrhages</td>
<td>125</td>
<td>9-04</td>
</tr>
<tr>
<td>Necrosis of the maxillary processes</td>
<td>25</td>
<td>1-8</td>
</tr>
<tr>
<td>Subepidermal blisters</td>
<td>180</td>
<td>13-0</td>
</tr>
<tr>
<td>Malformations of foot plates</td>
<td>68</td>
<td>4-9</td>
</tr>
<tr>
<td>Micromelia</td>
<td>130</td>
<td>9-4</td>
</tr>
</tbody>
</table>

* In many cases embryos showed several of the above abnormalities and so were recorded more than once.

Eighteen-day-old embryos. In the 407 embryos (from 104 pregnant females) in this group the frequency of those with deformations of limbs is lower than in the 14-day-old embryo group. There were forty-two embryos (10-3%) with micromelia (Plate 2, figs. C, D), but only twenty-four with abnormal digits (5-8%). The latter figure indicates that embryos with necrotic changes and with haemorrhages in the mesoblast of the foot plates probably do not survive from the 14th to the 18th day. On the other hand, cleft palate was found in 214 of these embryos (i.e. 52-7%). The appearance of the cleft palate was exactly like that shown in the paper by Fainstat (1954).

Light and electron microscopy of the abnormalities

The primary effect, i.e. the dilatation of the venous marginal sinus (Plate 2, figs. F, G), is more frequent in the hind-limb buds and is characterized by discontinuities in the endothelial lining of this blood vessel. The limb buds with

Plate 2

Fig. A. Experimental 14-day-old mouse embryo with subepidermal blister in the temporal region. Note also the malformation of the hind-foot plate. x 7.
Fig. B. Control 18-day-old mouse embryo. x 4.
Fig. C. Experimental 18-day-old mouse embryo with micromelia. x 4.
Fig. D. Necrotic area in the central part of the cartilage condensation of femur in a 14-day-old experimental embryo. x 110.
Fig. E. Necrosis of the central part of the third-toe cartilage condensation in 14-day-old experimental embryo. x 80.
Fig. F. Axial section through a control hind-limb bud (14-day-old mouse embryo). x 110.
Fig. G. Axial section through an experimental hind-limb bud with an extensive dilation of the marginal venous sinus. x 110.
a dilated sinus also sometimes show increased vascularization in other regions of the mesoblast.

At the electron-microscope level, the endothelial cells of dilated venous sinuses contain far more numerous Golgi groups with many primary and secondary lysosomes around them than are normally seen in the endothelial cells of untreated animals (Plate 3, figs. A, B). Some of these cells were found to contain extranuclear necrotic centres (Plate 3, fig. C).

Histological examination of the necrotically changed limb buds (secondary effects) showed that the injury is usually situated at the distal margin of the foot plate and only in a small proportion of cases was the necrotic area located in other than marginal parts of the limb mesoblast. Regardless of the location, such areas are as a rule sharply delineated from the healthy mesoblast tissue and represent virtually empty-looking spaces sparsely scattered with blood cells, cells containing pycnotic nuclei, necrotic cells and cell debris (Plate 3, figs. D, E).

In the extensive haemorrhages the shape of the affected limb buds appears to be abnormal, as the margin of such foot plates becomes thickened due to the presence of the haemorrhage. This is particularly clear in median sections, which appear to be much less pointed than those of the control embryos (Plate 3, fig. F). The haemorrhages consist of dense masses of blood cells (Plate 3, fig. G). The venous marginal sinus is usually absent in the areas occupied by extensive necrosis or haemorrhages. However, it must be pointed out that even in limb buds with extreme injuries of these types the epidermal covering is always intact and its cells appear to be completely unaffected (see Plate 3, figs. D, E, G).

Histological examination of the limb buds in 14-day-old embryos has also shown that, regardless of whether there were any macroscopically visible changes or not, most of the experimental limb buds that were examined contained necrotic areas inside the skeletal cartilage condensations (Plate 2, figs. E, F). These areas are found almost exclusively in the condensations of the limb skeleton and are very rarely encountered in the blastemata of other parts of the embryonic skeleton.

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**Plate 3**

Fig. A. Electron micrograph of a control endothelium cell fragment. ×18000.

Fig. B. Electron micrograph of an experimental endothelium cell showing abundance of Golgi groups with primary lysosomes in the vicinity. ×18000.

Fig. C. Experimental endothelium cell with the extranuclear necrotic centre (nc). ×5200.

Fig. D. Histology of the distal marginal necrotic area in the forelimb bud mesoblast. Note completely unaffected epiblast with the apical ectodermal ridge and the cell debris inside the necrotic area. ×85.

Fig. E. Histology of the distal marginal necrotic area in an experimental hind-limb bud mesoblast. Note the unaffected epiblast and the cell debris inside the necrotic area. ×105.

Fig. F. Extensive distal haemorrhage in an experimental forelimb bud. ×42.

Fig. G. Higher power showing the nucleated blood cells in the mesoblast haemorrhage. ×170.
Like the histological survey of the lesions, the electron-microscope examination confirmed that the epidermal covering in the necrotically changed limb buds or in those containing extensive haemorrhages consists of cells with the same ultrastructural features as those in control embryos (Plate 4, figs. A, B). The necrotic cells in the mesoblast adjacent to the necrotic areas or to the haemorrhages contain in the cytoplasm large lysosomes and cytolysomes, but the nuclei of these cells do not show any apparent abnormality (Plate 4, fig. C). Necrotic cells of the same type were found in the necrotic areas within the cartilage condensations (Plate 4, fig. D).

**DISCUSSION**

In animal experiments cortisone is known to cause a general retardation of embryonic development (Moscona & Karnofsky, 1960) and also to be specific in producing cleft palate (Walker & Fraser, 1957; Fraser, 1961). As far as hydrocortisone is concerned, it is undoubtedly also a very potent embryotoxic drug, although relatively non-toxic for adult animals, including pregnant females. The high embryotoxicity of hydrocortisone is further emphasized by the fact that, due to the partial barrier effect of the placenta, the mean value for hydrocortisone concentration in human blood is 10.8 µg/100 ml (Sweat, 1955), whereas in the human amniotic fluid it is only 2 µg/100 ml (Cope, Hurlock & Sewell, 1955). In other words, although the concentration of hydrocortisone behind the placenta is lower, the embryos become affected much more than the mothers.

On the whole, the embryotoxic effect of hydrocortisone in mice is very similar to that of cortisone on pregnancy in rabbits, as described by Courrier & Cologne (1951) and by DeCosta & Abelman (1952).

The present investigations on the embryotoxicity of hydrocortisone acetate have confirmed its relatively high cleft-palate specificity, as is the case with cortisone, and this is in agreement with the fact that cortisone is active

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**PLATE 4**

Fig. A. Electron micrograph showing a control limb-bud epiblast (E) and the underlying mesoblast cells (M) separated by the basal lamina (bl) and the reticulin layer (rl). × 5200.

Fig. B. Electron micrograph of an area similar to that in fig. A but with a necrotic region in the mesoblast after treatment with hydrocortisone. Note that the epiblast (E), together with the basal lamina (bl), remains unaffected, while the necrotic region is full of cell debris (cd). Also note change in the ultrastructure of the reticulin layer (rl). × 5200.

Fig. C. Extranuclear necrosis of a mesoblast cell from a region adjacent to the marginal necrotic area in the limb bud mesoblast. Note lysosomes (l) and vacuolation of the cytoplasm (v). × 6500.

Fig. D. Electron micrograph of an early cytoplasm necrosis of a chondroblast at the necrotic area within a toe-cartilage condensation. Note numerous Golgi groups (G) and abundance of lysosomes (l). × 13000.
pharmacologically after conversion into hydrocortisone (Cope, 1964). Virtually everything that is true for one of these hormones is true also for the other. In the mice in the present experiments, however, hydrocortisone acetate did not cause any noticeable retardation of growth, as judged by the average weights of 14-day-old experimental and control embryos.

The abnormalities described in this paper, such as dilatation of the marginal venous sinus in both fore- and hind-limb buds, necrosis of the distal portions of the limb mesoblast, distally located haemorrhages in the limb mesoblast and necrotic changes in the central portions of skeletal cartilage condensations, together with occasional micromelia, indicate that hydrocortisone is capable of changing the normal metabolic balance in the developing limb mesoblast. It must be stressed that similar abnormalities in limb development in the form of shortened humeri and femora and missing phalanges have been reported in the chick by Moscona & Karnofsky (1960). In general, the present investigations have shown that the mesoblast of the limb bud, particularly in its distal portion, is much more sensitive to hydrocortisone activity than is the epiblast, which remains virtually unaffected. Ragan et al. (1949) have also shown that the mesodermal components of healing wounds in rabbits are those which are inhibited after treatment with cortisone.

As to the mechanism and origin of these abnormalities it is feasible to assume that in all the observed cases the primary changes take place in the endothelial cells of the marginal venous sinuses. From electron-microscope examination it seems that injury to these cells involves in the first instance an overproduction of lysosomes and possibly at the same time an increased synthesis of those hydrolytic enzymes not contained in the lysosomes. At an early stage in cells where there was a very moderate dilatation of the marginal sinus there is an increase in the number of Golgi groups with primary and secondary lysosomes in their vicinity. When there are large numbers of lysosomes and they have increased in size to reach the diameter of cytolyosomes, the cells become necrotic. It must be remembered, however, that there are many authors who describe hydrocortisone as a stabilizing agent for lysosomes (de Duve et al. 1961; Jacobson, 1964). Necrosis of cells preceded by an increase in the number and size of lysosomes is confined at first to the cytoplasm of the affected cells, while their nuclei remain completely unaffected. Similar observations were made in experiments with chick embryos treated with thalidomide, but in that case the endothelium of the axial artery was the primary site of the injury (Jurand, 1966). Cellular necrosis and disruption of the endothelial lining of either arteries or venous sinuses leads most probably to disturbances of the local blood circulation.

Extensive well-delimited necrotic areas and haemorrhages in the distal portions of limb-bud mesoderm at the site of the marginal venous sinuses should be regarded as secondary changes caused by an inadequate blood supply due to impaired blood circulation. The necrotic areas in the central parts of limb cartilage condensations seem to be caused by the decrease in food and oxygen
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supplies due to their deep location within a tissue which itself is not vascularized. In circumstances in which there is a deficiency of food and oxygen, the innermost parts of an unvascularized cartilage will suffer first. On the other hand, the lesions in the central parts of cartilage condensations might be caused directly by the interference of hydrocortisone in the synthetic activity of the cartilage cells, for it is known that cortisone inhibits the synthesis of chondroitin sulphate (Layton, 1951; Boström & Odebald, 1953). This mechanism, however, could not explain why the necrotic areas are located exactly at the centres of the affected cartilage condensations.

Moscona & Karnofsky (1960) have reported defects in ossification of the long bones of the extremities in the chick after cortisone treatment which may have been caused by prior necrosis inside the cartilage condensations.

In attempting to interpret the mechanism of hydrocortisone embryotoxicity, it should be pointed out that in the more recent literature there are consistent indications that, on one hand, it acts as an anti-inflammatory drug and is protective against cell injury by various agents, but, on the other hand, it can also delay the repair of cellular injury. This paradoxical situation is probably the result of stimulation of DNA-dependent messenger RNAs and of other RNAs involved in the synthesis of specific proteins in general and enzymes in particular. The end result of such a stimulation will depend on whether it leads predominantly to cell repair, or whether the synthesis of deaminases and proteolytic enzymes is stimulated, when it will result in the delay of cell repair or even in cell injury and death.

Another feature of hydrocortisone injury is the occurrence of subepithelial blisters on the skin, accompanied occasionally by local necrosis. They occur preferentially in certain regions of the body, e.g. the temporal region on the head, the mandibular processes and, dorsally, on the proximal parts of the limbs. In some cases the regions seem to contain single-whisker primordia where an intensive protein synthesis probably takes place. This detail may be related to the growth-inhibiting syndrome after cortisone administration described by Karnofsky, Ridgway & Patterson (1951) in chick embryos, where complete inhibition of feather formation was observed.

In conclusion it should be pointed out that, although hydrocortisone is widely used in medicine and is regarded as a safe drug from the point of view of its side effects, nevertheless its embryotoxicity in animal experiments is quite considerable.

SUMMARY

Hydrocortisone acetate injected into mice subcutaneously (25 mg/kg) on the 10th, 11th and 12th days of pregnancy causes in the limb buds of 14-day-old embryos dilatation of the venous marginal sinuses (primary effect) (14.4%), extensive distal mesoblast necrosis (7.9%), distal haemorrhages (10.7%), and frequent necrotic centres in the limb skeleton blastemas (secondary effects).
Similar injuries are found in the tail tip. In some embryos the foot plates show deformations, possibly due to missing digits, which seems to be the effect of healing of previous injuries (tertiary effect).

Other macroscopically visible malformations included: micromelia in about 9.4% of embryos and necrosis of the maxillary processes with facial fissures (1.8%). In addition, the affected embryos show subepidermal blisters on the skin in the temporal region of the head and on the back. Cleft palate was found in 52.7% of 18-day-old embryos.

RESUME

L’effet de l’acétate d’hydrocortisone sur le développement de l’embryon de Souris

L’acétate d’hydrocortisone injecté aux Souris par voie sous-cutanée (25 mg/kg) le 10e, 11e et 12e jour de la gestation provoque dans les bourgeois de membres d’embryons de 14 jours une dilatation des sinus veineux marginaux (effet primaire) (14.4%), une nécrose étendue du mésoblaste distal (7.9%), des hémorragies distales (10.7%), et des centres de nécrose abondants dans les blastèmes du squelette de membre (effets secondaires). Des altérations identiques ont été observées à l’extrémité de la queue. Chez certains embryons, les palettes pédiéuses montrent des déformations qui sont peut-être dues à l’absence de doigts et vraisemblablement provoquées par la cicatrisation de lésions antérieures (effet tertiaire).

D’autres malformations sont visibles macroscopiquement: la micromélie chez 9.4% des embryons, la nécrose des bourgeois maxillaires avec des fissures faciales (1.8%). Les embryons affectés présentent en outre des ampoules sous épidermiques de la peau dans la région temporale de la tête et sur le dos. Des fissures palatines ont été observées chez 52.7% des embryons âgés de 18 jours.

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