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Histological study of timing and embryology of notochordal abnormalities in rat exposed *in utero* to Doxorubicin

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Summary. Experimental Doxorubicin-exposure *in utero* is correlated with foetal oesophageal atresia, tracheo-oesophageal fistula, axial alterations. While gastro-intestinal and respiratory defects have been largely investigated, only sporadic data have been published to date on notochordal and vertebral defects. The aim of this work was the study of the genesis of chordal and vertebral abnormalities in rat embryos and foetuses exposed to Doxorubicin and the study of their correlation with oesophageal and tracheal defects.

For this purpose, pregnant rats were i.p. injected with saline (control) or with 4mg/Kg b.w. Doxorubicin on days 9.5 and 10.5 of gestation. Embryos and foetuses were morphologically analysed on days 10.5-15 and 16, 18, 20 of gestation respectively, fixed in formaldehyde and histologically processed. Slides were routinely stained with haematoxylin-eosin (11-15 days *post coitum* embryos and all foetuses) or specifically stained with aniline blue for the staining of basal laminae (10.5 days *post coitum* embryos). Moreover, some foetuses at term (20 days *post coitum*) were processed for bone and cartilage staining.

The data obtained in the present work confirm the specificity of Doxorubicin in inducing gastro-intestinal and tracheal defects, describe the genesis of these defects step by step, describe the type and the genesis of notochordal abnormalities and their fate and exclude the role of Doxorubicin in inducing axial skeletal malformations.

Key words: Doxorubicin, Notochord, Abnormalities, Embryo, Rat

Introduction

Oesophageal atresia and tracheo-oesophageal fistula (Thompson et al., 1978; Diez-Pardo et al., 1996; Qi et al., 1996; Merei et al., 1997a, 1999; Possoegel et al., 1998; Xia et al., 1999; Zhou et al., 1999), skeletal or notochordal alterations (Merei et al., 1997b, 1998a,b; Kotsios et al., 1998; Possoegel et al., 1999; Qi and Beasley, 1999; Xia et al., 1999) have been described in foetuses or embryos exposed *in utero* to 1.75-2 mg/Kg b.w. Doxorubicin (Adryamicin, DOXO) from 6 to 9 days *post coitum* (d.p.c.). As far as the oesophageal abnormalities are concerned, a failure of the tracheal bud to develop normally from the primitive foregut has been described as the main event which leads to DOXO-related tracheo-oesophageal anomalies in rats (Merei et al., 1997a; Possoegel et al., 1998; Zhou et al., 1999). With regard to skeleton, notochordal abnormalities, observed at the middle of rat development (11.5-14 d.p.c.), have been described as position abnormalities, abnormal branching (duplications or triplications) or abnormal notochordal bending (Merei et al., 1997b, 1998a,b; Possoegel et al., 1999; Qi and Beasely, 1999) but a complete description of the fate of the atypical chordal material has never been done, nor has a direct correlation between chordal alterations and vertebral malformations ever been demonstrated. In Mammals, the notochord is present only in early stages of embryonic life. Its fundamental roles are to provide the cranio-caudal orientation of the embryonic axis and to act as primary organiser for adjacent developing organs. The development of the notochord has been extensively described by Jurand (1974) and by Barteczko and Jacob (1999). The notochordal formation is first detectable during gastrulation, when mesodermal cells are introflecting from Hensen's node. Initially (for rats, nearly in 9-d.p.c.-old embryos), the entire notochord is incorporated into the endoderm, forming the archenteron roof. Successively, the endoderm consolidates to form a continuous layer and the notochord starts to separate from the archenteron in a cranio-caudal direction, forming a dorsal elongated rod from Rathke's pouch to the posterior neuropore. Because of its transient nature, the organ itself disappears in rats on 16 d.p.c. chordal remnants co-operate in forming intervertebral discs and the basioccipital bone.

The aim of this work was the study of chordal and vertebral anomaly generation in rat embryos exposed to DOXO during the middle part of organogenesis and the correlation between chordal anomalies and tracheo-

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oesophageal defects. DOXO related abnormalities of endodermal derivatives have been described in our previous study, both in rat embryos analysed at midgestation and in rat foetuses at term (Menegola et al., 2001).

The second aim of the present study was the description of the morphological alterations of endodermal structures during the embryogenic and foetal periods after exposure to DOXO.

For these purposes, pregnant rats were intraperitoneally treated with DOXO during the critical notochordal development period (9.5 and 10.5 d.p.c.) according to protocol previously used in our study (Menegola et al., 2001). Embryos and foetuses have been analysed everyday during embryogenesis (10.5-15 d.p.c.) and at 16, 18 and 20 d.p.c.

Materials and methods

Crl:CD rats (Charles River, Calco, Italy), maintained in an air-conditioned room (T=22±2°C, Humidity= 55±5%) with 12 hours/day light cycles (6 a.m.: 6 p.m.), were used for this experiment. At the time of proestrus, females were caged with males overnight, and the day of positive vaginal smear was called day 0 of gestation.

Pregnant female rats were intraperitoneally treated with 4 mg/Kg b.w. of DOXO (gift of Pharmacia and Upjohn, Nerviano, Italy) on days 9.5 and 10.5 *post coitum*, according to the treatment regimen used in our previous study (Menegola et al., 2001). Controls were injected at the same time with saline. Females were sacrificed on days 10.5, 11, 12, 13, 14, 15 (embryonic period) or on days 16, 18, 20 *post coitum* (foetal period). Embryos or foetuses were collected and examined for external morphological alterations and degree of development. 50% of the 20-d.p.c.-old foetuses were randomly chosen and processed for skeletal examination according to the double staining method (alcyan blue for cartilage, alizarin red S for bone) of Kimmel and Trammel (1981). The other foetuses and all the embryos were fixed (4% formaldehyde) and some (about 10 conceptuses per group per day) were processed for histological examination. Technovit 8100 (Bioptica) or paraffin (Merk) were used for the inclusions. Haematoxylin and eosin staining was used for routine analysis, while aniline blue was used on 10.5-d.p.c. embryo sections for basal laminae staining, according to the method proposed by Grunz (1994).

At least 5 control and 5 DOXO-exposed females were sacrificed on days 10.5-18, and 10 females per group were sacrificed on day 20 *post coitum*.

Results

Tracheo-oesophageal development

Control

The original laryngotracheal outgrowth was visible on day 11 *post coitum* as a rounded ventral bud with an extensive communication with the ventro-caudal part of the pharynx. At 12 d.p.c., from this primordial outgrowth the trachea extended caudal, ventral to, and roughly parallel with the oesophagus, exhibiting a pair of knob-like enlargements (the primary bronchi). During the successive days, the trachea elongated and bronchi branched and re-branched to form the branchial tree and to differentiate the lung lobules (Fig. 1g). Only the epithelial lining and the tracheal glands were derived from the original endodermal outgrowth from the pharynx. The cartilage, connective tissue and muscle of its wall were, in fact, formed by mesenchyme massed about the growing endodermal tube.

DOXO 4 mg/Kg/day

After the DOXO exposure, 10.5-day-old embryos showed a disorganised digestive epithelium, characterised by diffuse cellular death (Fig.1b). 12 hours later (11 d.p.c.-old embryos) the digestive epithelium damage was no longer visible and histologically the foregut appearance was similar to controls (Fig. 1d). Foregut alterations, however, were visible again from day 12 *post coitum* on. At 12 d.p.c., in fact, the laryngeal ventricles appeared reduced and characterised by disorganised epithelium (Fig. 1f) and, below, an abnormal opening between the trachea and the oesophagus was detectable. In particular, the digestive and respiratory tubes appeared unseparated, forming an eight-shaped structure continuing below with a blind pouch, identified as the atresic oesophagus. As far as the more ventral tube was concerned, the first tract appeared with clear tracheal characteristics (and was also able to form the main bronchi), but caudally continued into the stomach (Fig. 1h). This complex structure was classified as a tracheo-oesophageal fistula with oesophageal atresia (Fig. 2b, 3b). In some cases (nearly 30%), this feature was persistent until term of gestation, while, in the other cases, in 14 d.p.c. embryos, the oesophageal pouch appeared resorbed (Fig. 2c, 3c) and, from 16 d.p.c. on, in nearly the 50% of the examined foetuses, only the bronchial tree was visible (Fig. 2d, 3d).

Chordal and vertebral development

Control

In 10.5 d.p.c. old rat embryos, the chordal rod appeared separated from the foregut endoderm, occupying an intermediate position between the neural and the digestive tubes (Fig. 1a). After staining the basal laminae, using the aniline blue staining method, the three cellular layers resulted effectively well separated (Fig. 4a,c). Later on development, mesenchymal cells penetrated between the digestive tube and the notochord (from day 11 on in a cephalic-caudal direction) (Fig. 1c, 4e) and between the neural tube and the notochord (from day 12 on, in a cephalic-caudal direction). The

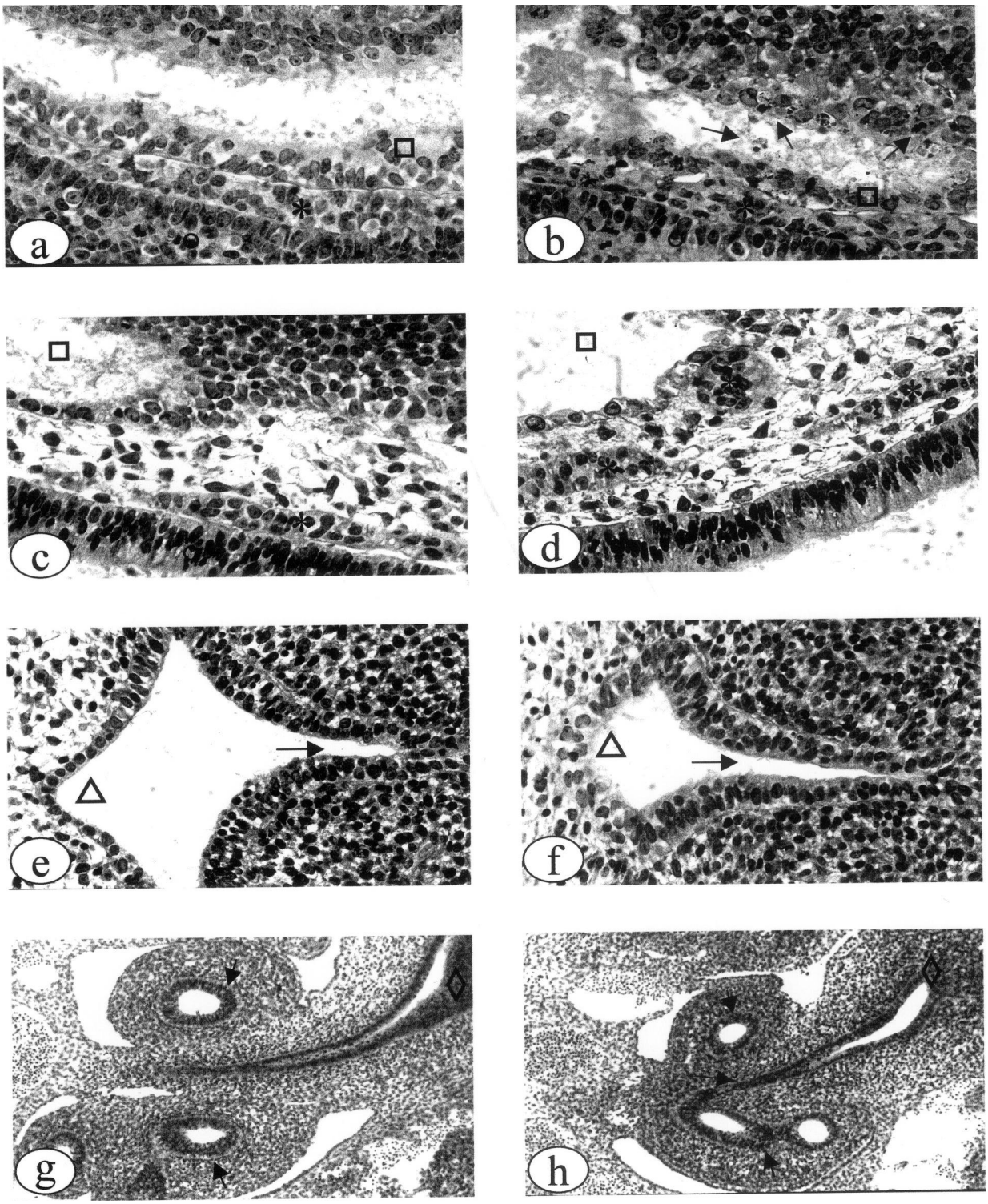


Fig. 1. Tracheo-oesophageal development in controls **(a, c, e, g)** and the genesis of tracheo-oesophageal fistula in DOXO-exposed embryos **(b, d, f, h)**. **a and b.** 10.5 d.p.c-old embryos: parasagittal sections. The neural epithelium (circle), chordal tissues (asterisk), and digestive epithelium (square) are clearly recognisable both in the control embryo **(a)** and in the DOXO-exposed embryo **(b)**. In the embryo exposed to DOXO, however, note the clearly disorganised digestive epithelium with a number of detectable picnotic nuclei (arrows). **c and d.** 11-d.p.c-old embryos: parasagittal sections. Circle: neural epithelium; asterisk: notochord; square: pharyngeal lumen. Both in the control **(c)** and in the DOXO-exposed embryo **(d)** the mesenchymal tissues separate the digestive tube from the notochord. No damage is visible in the DOXO-exposed embryo at the level of the endodermal tissue. In **d** note the abnormally shaped notochord, forming a bend to reach the digestive epithelium. **e and f.** 12-d.p.c-old embryos: cross sections at the level of the pharynx. In the control embryo **(e)** the pharynx (triangle) is clearly enlarged laterally, forming the laryngeal ventricles, and ventrally, forming the tracheal bud (arrow). In the DOXO-exposed embryo **(f)**, at this histological level, the tracheal bud appears quite normal (arrow) but a clearly reduced pharyngeal lumen is visible (triangle), characterised also by an undifferentiated epithelium. **g and h.** 13-d.p.c-old embryos: frontal sections at the level of the main bronchi (white arrows) and of the stomach (rhombus). In **g** (control) note the oesophageal structures (epithelium and mesenchyme) anatomically well separated from the bronchial apparatus. On the contrary, in **h** (DOXO-exposed embryo) note the communication between the oesophageal and bronchial epithelia (black arrow). Also the mesenchyme around the epithelia appears not to be separated. a-f, x 400; g, h, x 100

mesenchyme surrounding the notochordal rod (the sclerotome), from 14 d.p.c. on, condensed and differentiated forming the vertebral tissues (Fig. 3a). At the same time of the sclerotome condensation, the notochord started to regress: at 16 d.p.c. the notochord showed the typical rosary-shape (Fig. 7a), while at 18 and 20 d.*p.c.*only inter-segmental remnants were visible (Fig. 7b).

DOXO

At 10.5 d.p.c. the notochord appeared to be still incorporated into the foregut endoderm (Figs. 1b, 4b). The continuity of the basal laminae of these tissues was clearly shown by the aniline-blue staining method (Fig.

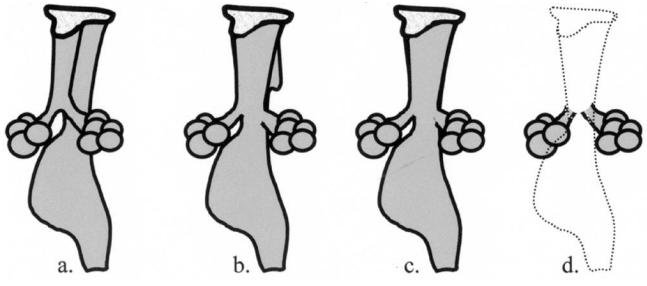


Fig. 2. Scheme illustrating the tracheo-oesophageal structure appearance from 14 d.p.c. on in controls **(a)** and in DOXO-exposed conceptuses **(b-d)**. In controls **(a)** two separated tubes originate from the pharynx: the trachea, continuing with the bronchial tree, and the oesophagus, continuing into the stomach. As far as DOXO-exposed conceptuses are concerned, in some cases the tracheo-oesophageal fistula with oesophageal atresia **(b)** is observable from 12 d.p.c. until term; in other samples a completely atrophic oesophagus can be identified at 14 d.p.c. **(c)** and, from 16 d.p.c. on only the bronchial tree is visible in the most seriously affected foetuses **(d)**.

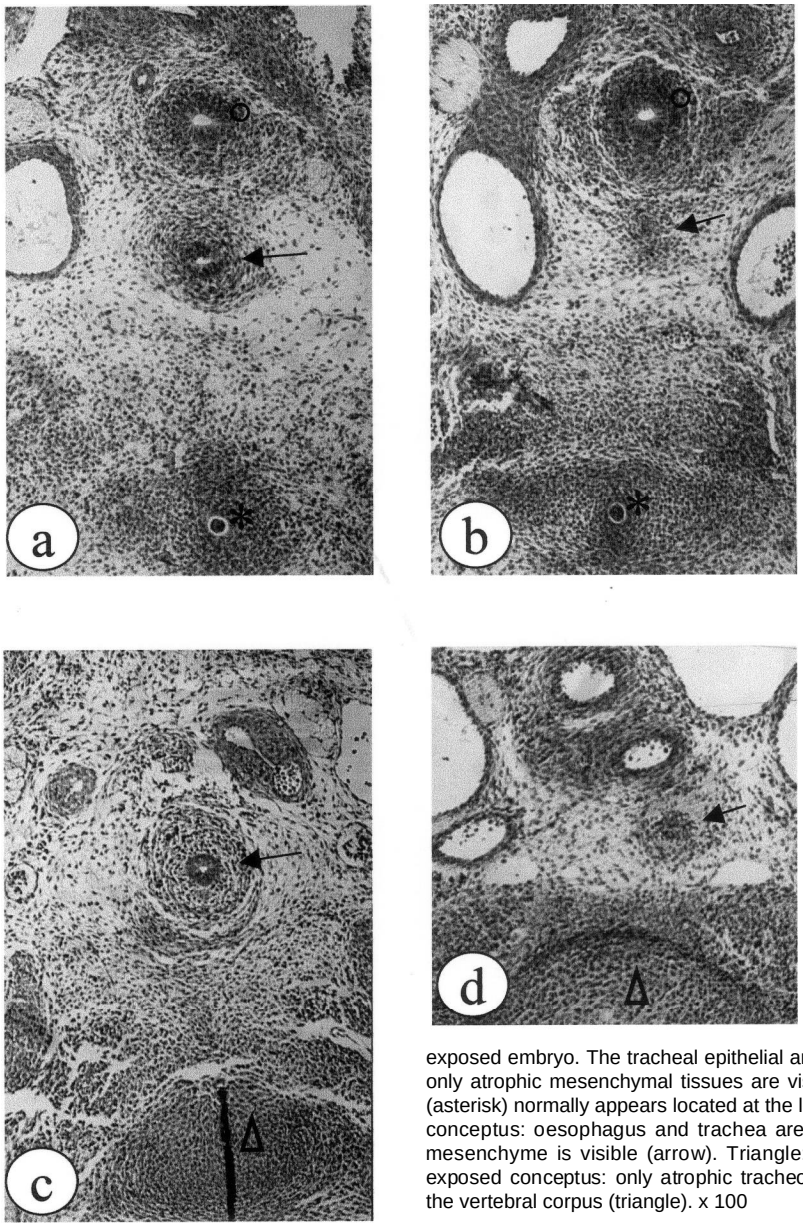


Fig. 3. Different types of tracheo-oesophageal alterations in DOXO-exposed conceptuses. Cross sections. **a.** 14-d.p.c-old control embryo: the two distinct tracheal (circle) and oesophageal (arrow) structures are visible: note the epithelial structures surrounded by condensed mesenchymal layers. Asterisk: the notochord surrounded by the condensed sclerotome. **b.** 14-d.p.c.-old DOXO-exposed embryo. The tracheal epithelial and mesenchymal structures (circle) are similar to controls but only atrophic mesenchymal tissues are visible at the level of the oesophagus (arrow). The notochord (asterisk) normally appears located at the level of the sclerotome mass. **c.** 15-d.p.c.-old DOXO-exposed conceptus: oesophagus and trachea are indistinguishable: a unique epithelial tube surrounded by mesenchyme is visible (arrow). Triangle: the developing vertebral corpus. **d.** 16-d.p.c.-old DOXO-exposed conceptus: only atrophic tracheo-oesophageal remnants (arrow) are observable ventrally to the vertebral corpus (triangle). x 100

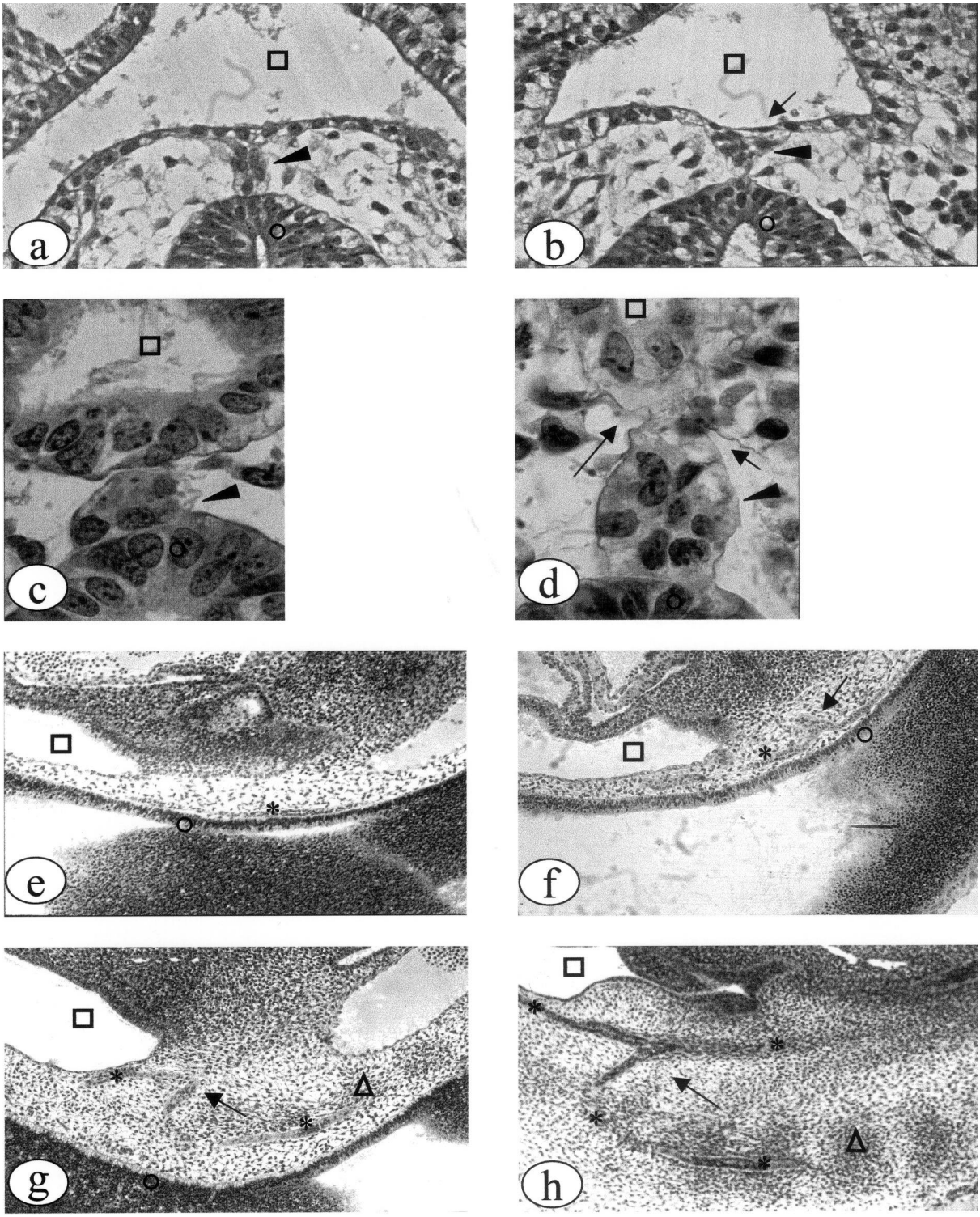


Fig. 4. Notochordal development in controls **(a, c, e)** and the genesis of chordal abnormalities in DOXO-exposed embryos **(b, d, f, g, h). a-d.** 10.5-d.p.c.-old embryos: cross sections at the level of the pharynx stained according to Grunz (1994) to show the basal laminae. In controls **(a, c)** the notochordal material (arrowhead) occupies an intermediate position between the pharynx (square: pharyngeal lumen) and the neural tube (circle). The notochordal basal lamina appears to be well separated both from the neural epithelium and from the foregut epithelial layer. On the contrary, in DOXO-exposed embryos **(b, d)** a continuous foregut and notochordal cellular layer is observable, as indicated by the continuity of the basal lamina (arrows). Square: pharyngeal lumen; circle: neuroepithelium; arrowhead: notochord. **e and f.** 11 d.p.c. old embryos. Parasagittal sections at the level of the foregut. Square: pharyngeal lumen; circle: neural tube; asterisk: notochord. The notochordal progression is linear in the control **(e)**, while a clear bending is visible in the DOXO-exposed embryo (**f**, arrow). **g and h.** DOXO-exposed embryos: parasagittal sections at the level of the foregut. Note the typical notochordal bending (arrows) visible in the 12-d.p.c.-embryo **(g)** and deeper in the 13-d.p.c.-embryo **(h)**. Square: pharyngeal lumen; circle: neural tube; triangle: sclerotome; asterisk: notochord. a, b, x 400; c, d, x 1,000; e-h, x 100

4b,d). The repair of endodermal damage, observed at 11d.p.c. (Fig. 1d), allowed the notochord to separate completely from the middle and caudal digestive tube, but not from the foregut. At this level, a bending of the notochord was observed from 11 d.p.c. on (Figs. 1d, 4f), becoming deeper and deeper on days 12 (Fig. 4g) and 13 (Figs. 4h, 5a) till 14 and 15 d.p.c., when different bending shapes were visible (for the detailed description see Fig. 6) (Fig. 5b,c). During the intra-segmental regression of the notochord, the bending structures regressed too, so no differences between controls and DOXO-exposed foetuses were detectable from 16 d.p.c. on (Fig. 7a,b). Also the vertebral structure appeared normal in DOXO foetuses at term (Fig. 7c).

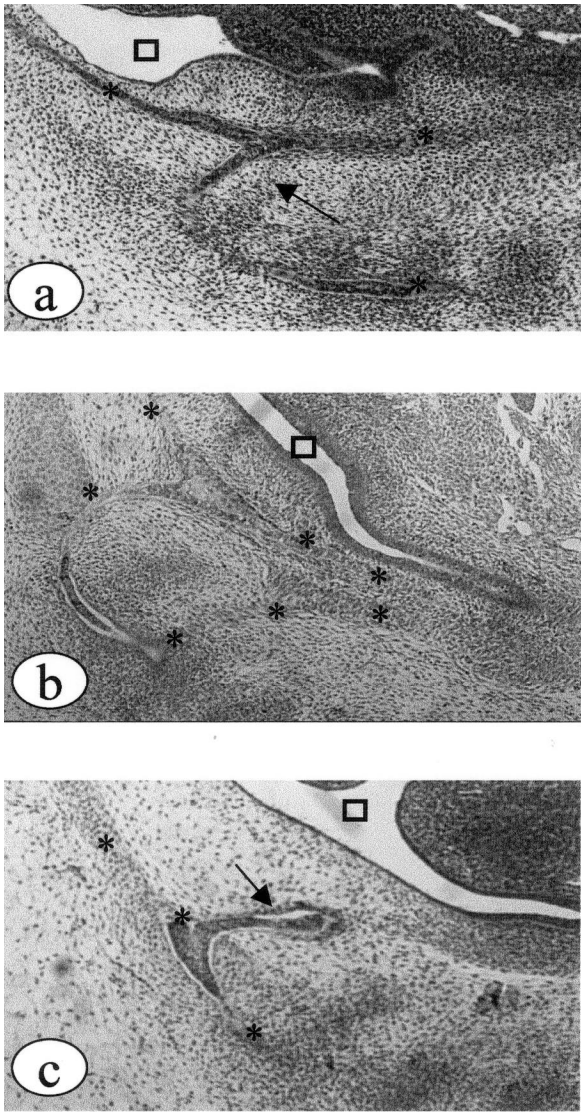


Fig. 5. Histological aspect of typical chordal abnormalities observed in DOXO-exposed conceptuses. Parasagittal sections at the level of the foregut. Square: pharyngeal lumen, asterisk: notochord. **a.** 13-d.p.c.-old DOXO-exposed embryo. Note the tight bending (arrow). **b.** 14-d.p.c.-old DOXO-exposed embryo. Along the notochordal course a ring-shaped structure appears. **c.** 14-d.p.c.-old DOXO-exposed embryo. A case of spine (arrow) emerging from the notochord in correspondence to the pharynx. x 100

Discussion

Tracheo-oesophageal abnormalities, observed in the present work after DOXO exposure, confirm literature data on rat embryos and foetuses exposed to this drug (Thompson et al., 1978; Diez-Pardo et al., 1996; Qi et al., 1996; Merei et al., 1997a, 1999; Zhou et al., 1999; Xia et al., 1999; Menegola et al., 2001). Interestingly, our data showed endodermal cellular damage in 10.5 day-embryos (i.e. 36 hours after the first treatment), while morphological abnormalities were detectable only 36 hours later (12 d.p.c.).

As far as the notochord is concerned, the continuity of the notochordal and endodermal basal laminae, observed at stages immediately after DOXO exposure (10.5 d.p.c.), seemed to be a developmental delay, unable to be efficacely recovered. At such an early stage, no description of embryonic structures after DOXO

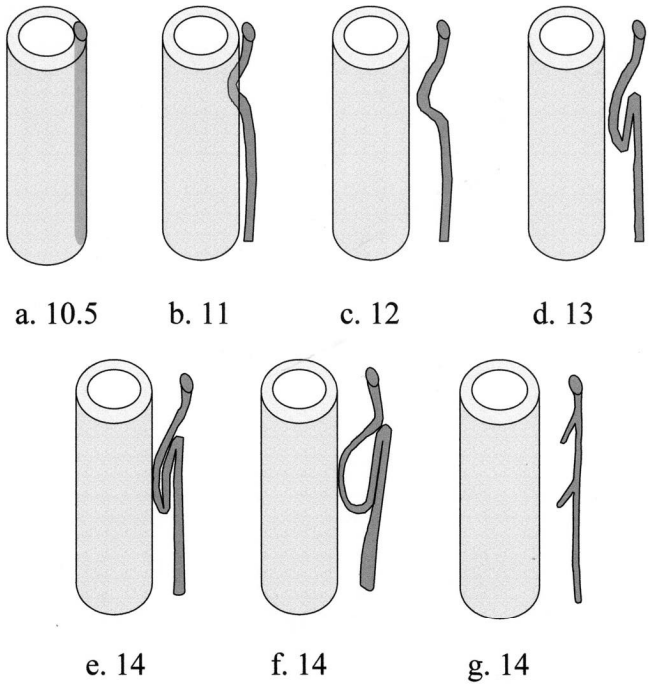
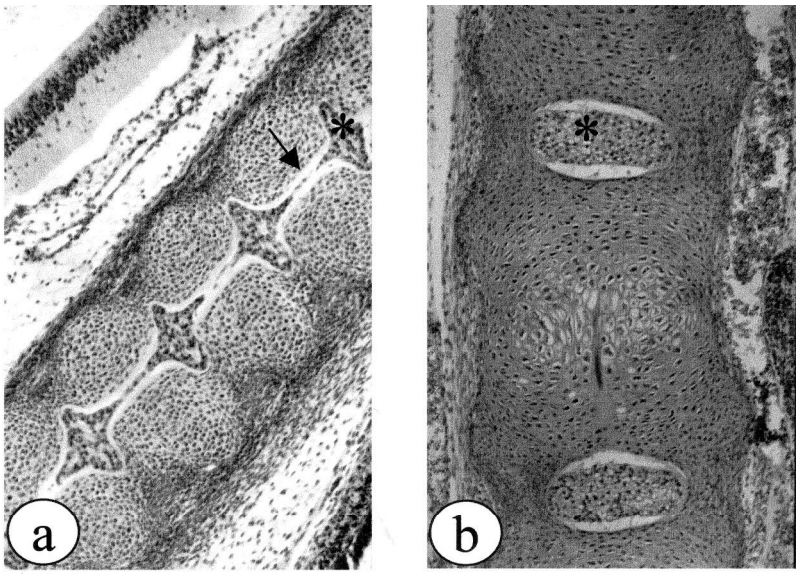


Fig. 6. Scheme illustrating notochordal abnormality in DOXO-exposed embryos from 10.5 to 14 d.p.c. **a.** 10.5-d.p.c.-old embryos: the notochord is still incorporated in the digestive tube roof. **b.** 11-d.p.c.-old embryos: only at the level of the pharynx a contact between endodermal and notochordal layers is visible and determines the formation of notochordal bending. The growth of the embryo and the elongation of the digestive tube pulls the notochordal ventrally and caudally, determining the deep bending on 12- and 13-d.p.c.-old embryos **(c, d)** and the formation of the typical chordal abnormalities observed on 14-d.p.c. DOXO-exposed embryos (**e**: thigh bending; **f:** ring; **g:** chordal spines) visible till 15 d.p.c. After the reorganisation of the notochordal material and the formation of the vertebral structures, abnormalities are no longer visible at the axial structures.

exposure has ever been done in literature.

At later stages (11 d.p.c. on), notochordal alterations were previously imprecisely described by several authors (Merei et al., 1997b, 1998a; Possoegel et al., 1999; Qi and Beasely, 1999) as position abnormalities and abnormal branching (duplications or triplications). Our observation of both cross-sections and parasagittal sections confirmed the diagnosis of notochordal bendings, described by Merei and colleagues (Merei et al., 1998b). In our present work, moreover, a complete description of the fate of this atypical chordal material was done and revealed no permanent damage at the axial structures. Other authors (Kotsios et al., 1998; Xia et al., 1999) reported skeletal malformations in rat foetuses exposed to DOXO: bilobed, hemi-ossified or unossified vertebral centra (Kotsios et al., 1998); lack of ossification; butterfly or hourglass-shaped vertebral centra; and hemi-ossified centra (Xia et al., 1999). These kinds of abnormalities should, more properly, be classified as variants or minor anomalies, because it is well known that they do not represent permanent damage, often related to a delay in ossification.

In conclusion, our findings suggest: 1) the endoderm is the specific embryonic target tissue for DOXO, as suggested also in our previous published work (Menegola et al., 2001); 2) insufficient repair mechanisms are involved in genesis of the malformations at the digestive tube: in 11-d.p.c.-old embryos, in fact, no morphological differences were observed between control and DOXO-exposed embryos



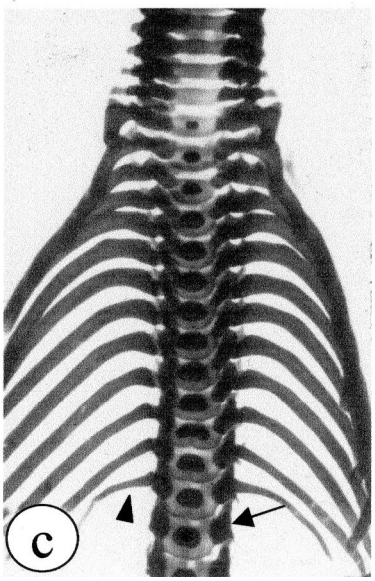


Fig. 7. The vertebral centra appears normal in DOXO-exposed foetuses. **a and b.** Parasagittal sections at the level of vertebral axis in 16- **(a)** and 18 **(b)**-d.p.c. DOXO-exposed conceptuses. In **a** note the characteristic rosary-shape of the notochordal tissue, identical to controls: the notochord regresses at the level of the vertebral centra (arrow) and persists at the level of nuclei pulposi (asterisk). In **b** only the intersegmental portions of the notochord are visible (asterisk). **c.** 20-d.p.c.-old foetus stained in toto for bone and cartilage, according to Kimmel and Trammel (1981). Asterisk: vertebral centrum, arrow: vertebral arches, arrowhead: rib. a, b, x 100; c, x 6

but the repaired digestive tube was unable to correctly differentiate later in development; 3) morphological notochordal abnormalities, observed at early stages, are probably a secondary consequence of endodermal decompaction. The formation of bendings, at the level of the notochordal length, could be, on the other hand, directly related with the delay in the separation of the notochordal material from the endoderm of the foregut, and the overgrowth of the foregut could be the cause of the appearance of these bendings (due to the indirect pulling of the notochord ventrally and caudally too); and 4) under our experimental conditions, the formation of bendings seems unable to influence normal sclerotome differentiation in rats, and regressed with the physiological elongation processes of the embryo. However, as almost nothing is known about rat and other species DOXO kinetic differences and about drug concentrations in embryos under different experimental conditions, our results can not be generalised for other species or other experimental regimens.

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