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Histological examination of major craniofacial abnormalities produced in rat foetuses with a variety of retinoids

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SUMMARY

Retinoic acid is a vitamin-A derived compound that plays an important role in craniofacial development. However, numerous studies have shown that exogenously administered retinoic acid analogues have teratogenic effects both in humans and in experimental animals. This paper presents the results of a study where exogenous administration of retinoic acid analogues to pregnant Wistar rats resulted in major craniofacial malformations of most of the foetuses. Seven groups of pregnant rats were exposed to a variety of retinoic acid analogues, administered in different doses on different gestational days. A further control group was not exposed to these compounds. All the rats were impregnated and sacrificed on the same gestational days (GD 0 and GD 18 respectively). Following examination of the 34 foetuses obtained from the retinoic acid-treated litters, 5 were chosen for histological investigation to describe the major, external craniofacial malformations resulting from exposure to the teratogens. These malformations included exophthalmos, anophthalmos, exencephaly, craniofacial asymmetry and facial tags. Also reported here are the incidences of teratomas and absorptions in these litters. Previously, we have reported the incidence of cleft lip and palate in rats subjected to an identical experimental regimen. Our data provide further evidence that retinoic acid analogues, all-trans-retinoic acid in particular, disturb normal craniofacial development in such a way that a spectrum of craniofacial malformations can be induced by embryonic exposure to these compounds.

Key words: Craniofacial development – Rat foetuses – Retinoids – Teratology

INTRODUCTION

The development of the craniofacial structures within the developing embryo is a complex process requiring a highly co-ordinated series of proliferative and morphogenetic events. These include the migration, proliferation and differentiation of neural crest cells (Emmanouil-Nikoloussi et al., 2000a), epithelial-mesenchymal interactions (Wedden, 1991) and extensive re-modelling of the extracellular matrix (Moxham, 2003). These events result in the formation of the individual components of the face and head, and in their timely unification, to produce a craniofacial complex that is structurally and functionally effective (Jacobsson, 1997). These processes

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are in turn regulated by molecular genetic events and cell signalling molecules (Meikle, 2002).

Over recent years, the rapid advances made in research techniques in the fields of genetics, molecular biology, biochemistry and immunology have resulted in a wealth of information and literature regarding the development of the craniofacial complex. Indeed, Sperber (1992) referred to craniofacial embryogenesis as "an increasingly tangled thicket of biochemical interactions". This complexity opens the way for a variety of interruptions to the developmental process that subsequently result in an array of craniofacial malformations. These vary from the prenatally fatal (e.g. gross/major craniofacial anomalies) to the functionally and aesthetically insignificant (e.g. bifid uvula) (Emmanouil-Nikoloussi and Kerameos-Foroglou, 1993).

In order to study both normal and abnormal craniofacial development, a variety of animals have been used, including rats (e.g. Klug et al., 1989), mice (e.g. Grant et al., 1997), chicks (e.g. Wedden, 1991) and even alligators (e.g. Ferguson, 1981). Despite the obvious differences in adult facial form compared with humans, Young et al. (2000) state that "there is an astounding conservation in the molecular specification and assembly of the embryonic facial structures". Thus, such animal models have been used extensively for the investigation of teratologically induced craniofacial malformations that can then be related to human craniofacial development (Johnston and Bronsky, 1991). These teratogenic agents act in specific ways on the developing cells and tissues to initiate abnormal embryogenesis (Jacobsson, 1997).

One group of significant teratogenic agents are the vitamin-A derived compounds known as retinoids, of which retinoic acid (RA) is probably the most important (Richman, 1992; Morriss-Kay, 1993). These are essential for embryogenesis, growth and differentiation but have also been shown to have wide-ranging teratogenic effects in different forms and doses, and at different stages of development (e.g. Webster and Ritchie, 1991; Emmanouil-Nikoloussi et al., 2000b, 2000c; Gunston et al., 2005). Emmanouil-Nikoloussi et al. (2003) have reported that neural tube defects, cleft palate and craniofacial dysmorphism are among the most common major anomalies produced as a result of foetal exposure to retinoid compounds.

Relatively few studies have reported, and described histologically, the induction of gross/major craniofacial abnormalities following the experimental administration of retinoids to animal models. This study describes major craniofacial abnormalities induced by treatment of pregnant female Wistar rats with a variety of retinoid analogues administered in different doses on different gestational days. Our findings are of value in increasing our understanding of the effects of experimental administration of retinoid analogues upon the development of the craniofacial structures. The presentation of cleft lip and palate in specimens exposed to an identical dosage regimen has been reported, and discussed, by Gunston et al. (2005).

Materials and Methods

The research presented in this paper was conducted courtesy of the Laboratory of Histology, Embryology and Anthropology, Faculty of Medicine, Aristotle University of Thessaloniki, Greece. All procedures were carried out under licensing rules according to the Helsinki Guidance for Animal Practice.

Twenty-two female Wistar rats, each 3 months of age and weighing 200-250g, were mated overnight. The presence of a copulatory plug the next morning was used to indicate gestational day zero (GD0). All pregnancies were "*prima gravida*". The rats were then housed in isolation with access to food and water "*ad libitum*" and kept under controlled conditions of temperature (20ºC ± 2ºC) and lighting (12 hour light/dark cycle). They were then grouped according to the RA analogue, dosage and days of treatment to be used (Table 1).

Corn oil suspensions of the RA analogues were prepared in dark-light conditions and stored in dark glass vials. A uniform suspension of each preparation was obtained by son-

Table 1. Protocol for RA administration to experimental animals.

GROUP	DAYS OF TREATMENT	RETINOIC ACID	DOSAGE mg kg^{-1} bw
A	8, 9, 10, 11	all-trans RA	30
B	9, 10, 11	all-trans RA	50
C	9.5, 10.5	all-trans RA	100
D	8, 9	all-trans RA	100
E	9, 10, 11, 12	13-cis-RA	30
F	9, 10, 11, 12	13-cis-RA	30
G	8, 9, 10, 11	acitretine	20
Control	Untreated		

icating and vortexing the solutions. The rats were fed the RA in corn oil preparation via gastric intubation under mild ether sedation. The precise dosage administered to each pregnant rat was calculated per kg body weight. The rats were then closely observed for toxic effects of RA, such as weight loss, hair loss, haemophthalmos (blood filled eyes) and vaginal bleeding (indicative of miscarriage).

At GD18, the pregnant rats were sacrificed by cervical dislocation. A perpendicular laparotomy was performed to fully expose the uterine horns. These were opened and the foetuses removed. Following removal of the foetal membranes, the foetuses were washed in phosphate-buffered saline (PBS; 10mM phosphate, 2.7mM KCl, 137mM NaCl, ph 7.4). Photographs were taken of the foetal heads under a stereomicroscope prior to histological preparation. The foetal heads were then microdissected under the stereomicroscope and fixed in 10% neutral formalin (phosphate-buffered). After embedding the foetal heads in paraffin wax, serial frontal sections were cut at 7 μm using an LKB Historange rotary microtome. Sections were then mounted onto glass microscope slides (coated with glycerine and albumin to aid adhesion), deparaffinized in xylene and rehydrated through a graded alcohol series. Finally, the sections were stained using Haematoxylin and Eosin, Toluidine Blue or Masson's Trichrome according to standard histological procedures and covered with cover slips using DPX solution. The completed sections were viewed with a Leica Leitz DM RB microscope and micrographs were taken using a digital camera and Image Grabber PCI software.

Results

The protocol of administering RA isomers in different doses on different gestational days had a profound effect upon the craniofacial development of the treated litters. Of initial significance is the incidence of teratomas and absorptions encountered in the RA-treated litters. These were not observed in the control group (Table 2). Group A (30 mg kg^{-1} bw all-trans RA, GD 8, 9, 10, 11) was the most seriously affected, with the litter consisting of 9 absorptions, 1 teratoma and a single foetus. Group G (20 mg kg^{-1} bw Acitretine, GD 8, 9, 10, 11) fared little better with the litter consisting of 10 teratomas and 2 foetuses (although with no absorptions).

From the study as a whole, five foetuses were selected that demonstrated the severe, externally observed, craniofacial malformations observed in the RA-treated litters. These abnormalities were identifiable both by macroscropic, morphological assessment and by histological examination (Figures 2-6). Table 3 summarises the findings of this study.

It is important to note that, alongside the major craniofacial malformations observed in these foetuses, foetuses from all the RA-treated groups demonstrated cleft lip and palate. This finding has previously been reported by Gunston et al. (2005) and will therefore not be discussed further in the context of this paper.

All our results were assessed in comparison with the untreated control group as this material was cut and prepared on the same gestational day as the RA-treated material (GD18). In addition, the control heads were deemed to have developed normally by reference to Kaufmann's "Atlas Of Mouse Development" (1999).

Table 2. Foetuses, teratomas and absorptions encountered in litters in experimentally RA-treated and untreated/control groups.

GROUP	FOETUSES	TERATOMAS	ABSORPTIONS
A	1	1	9
B	5	4	2
C	6	3	4
D	3	0	4
E	10	0	0
F	7	3	0
G	2	10	0
Control	6	0	0

Table 3. Gross external craniofacial abnormalities observed per group.

		GROUPS					
		A	B	C	D	E&F	G
EXOPHTHALMOS	Unilateral				+		
	Bilateral	+	+				+
ANOPHTHALMOS	Unilateral				+		
	Bilateral			+			
EXENCEPHALY		+	+		+		
GROSS ASSYMETRY				+			
FACIAL TAGS				+			+

Control Group

No abnormalities of the control heads were observed either macroscopically, or by morphological assessment or histological examination. This also applied to foetuses from a control litter where the pregnant dam had been fed corn-oil (but without RA) and in the same manner as the experimental animals. Figure 1 illustrates the stage of craniofacial development of a control specimen at GD18. The foetus demonstrated well-organised cranial coverage of bone, normal development of the eyes, craniofacial symmetry, an absence of facial tags and normal palatal development (Figure 1).

Group A (30 mg kg^{-1} bw all-trans RA, GD 8, 9, 10, 11)

This specimen presented with bilateral exophthalmos and ablepharia (i.e. absence of, or reduction in the size of, the eyelids). It also showed exencephaly. The roof of the skull was covered by a thick layer of connective tissue and externally by an epithelial skin layer. The appearance of the skull in the frontal area is a typical example of macrocephaly. Both eyeballs were smaller than those of the control specimen and cleft palate was present (Figure 2).

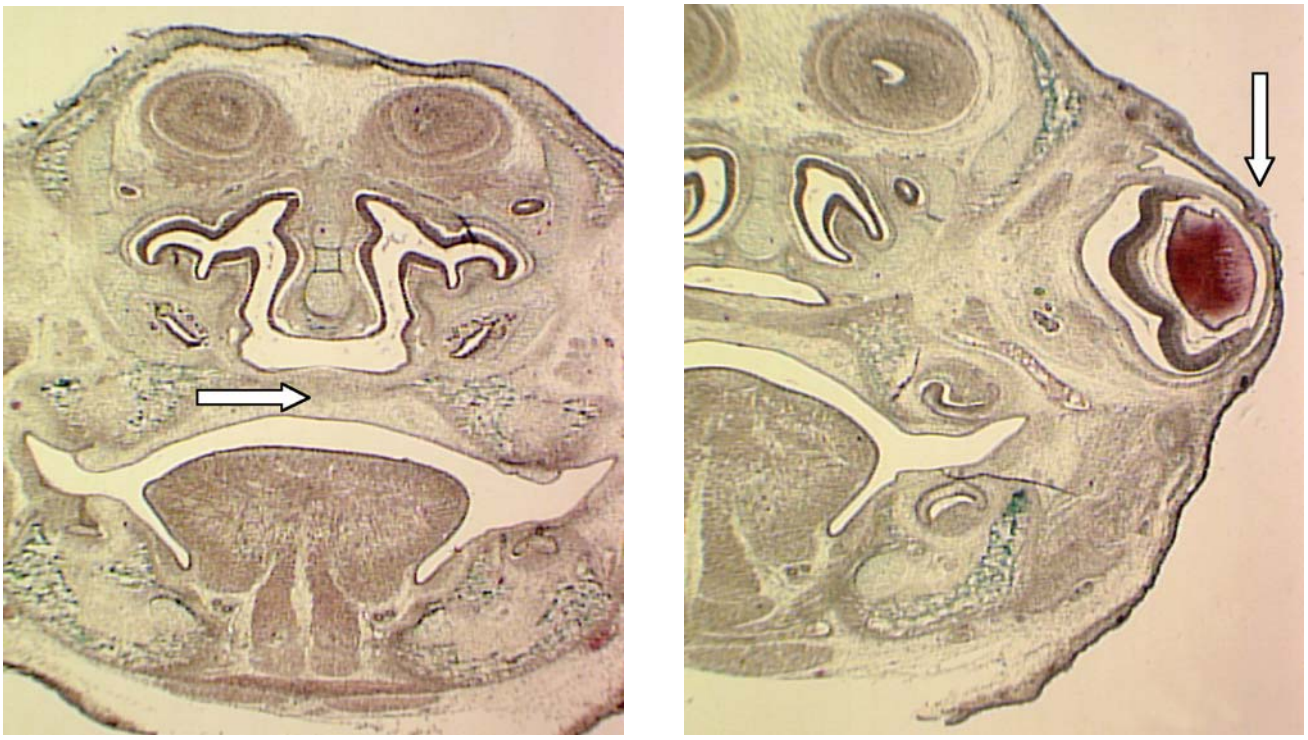
Group B (50 mg kg^{-1} bw all-trans RA, GD 9, 10 , 11)

This specimen had a similar appearance to that of the previous group, demonstrating bilateral exophthalmos with ablepharia and exencephaly. The roof of the skull was covered by a very thin layer of skin that in turn covered a thin layer of connective tissue. Microphthalmos was also present with distinct areas of corneal hyperplasia. Compared with the Group A specimen, the eyelids were slightly more developed and there was less protrusion of the eyeballs (Figure 3).

Group C (100 mg kg^{-1} bw all-trans RA, GD 9.5, 10.5)

This severely malformed specimen demonstrated gross craniofacial asymmetry with bilateral anophthalmos. Bilateral maxillofacial synostosis with small maxillary apophyses and palatal clefts was visible. There was also some evidence of arrested development of the lens

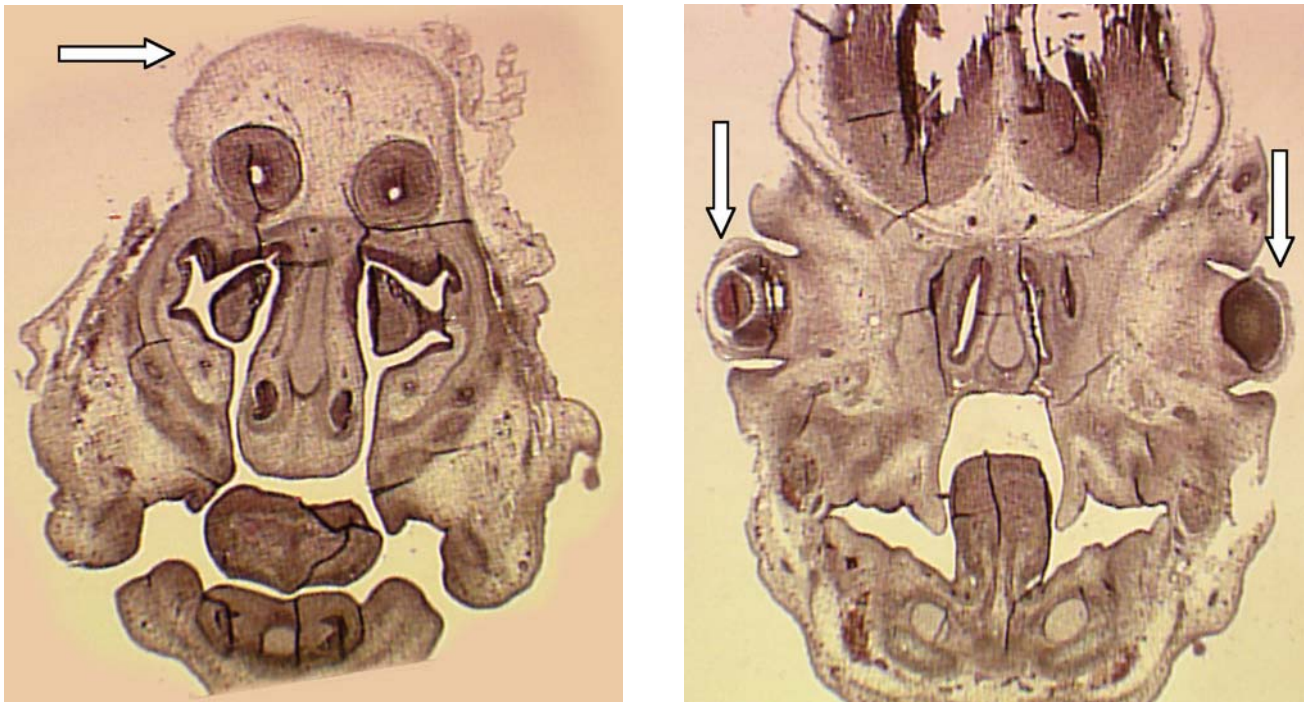
Figure 1. Control (untreated), at GD18.
A and B - coronal sections illustrating normal craniofacial development of a control specimen at GD18. (H & E stain; x 10).



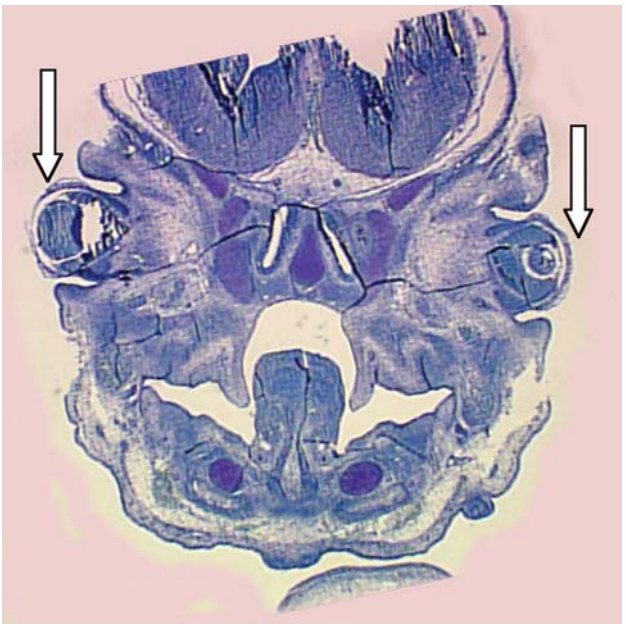
A **B**

Note that the palatal shelves have elevated and fused as indicated by the white arrow (although the nasal septum has yet to fuse with the palate; Figure 1A). The eye shows normal development with an intact, full coverage by the eyelids as indicated by the white arrow (Figure 1B). The developing brain is internalised and is covered by developing bone.

Figure 2. Group A (30mg kg^{-1} bw all-trans RA administrated GD 8, 9, 10, 11), at GD18.



A **B**



C

A - anterior coronal section showing exencephaly as indicated by the white arrow (H & E stain; x 10). B – coronal section showing bilateral exophthalmos and ablepharia as indicated by the white arrows (H & E stain; x 10). C -coronal section showing bilateral exophthalmos as indicated by the white arrows (Masson's Trichrome stain; x 10).

placode. In the connective tissue over the developing cranium, a considerable number of large blood vessels were observed and the developing dentition showed variation between the right and left sides (Figure 4).

Group D (100 mg kg^{-1} bw all-trans RA, GD 8, 9)

This specimen demonstrated unilateral exophthalmos with ablepharia (right side), unilateral anophthalmos (left side) and exencephaly. Bone formation was present only in a small lateral area of the cranium on the right side. The exophthalmic right eye also showed microphthalmia (Figure 5).

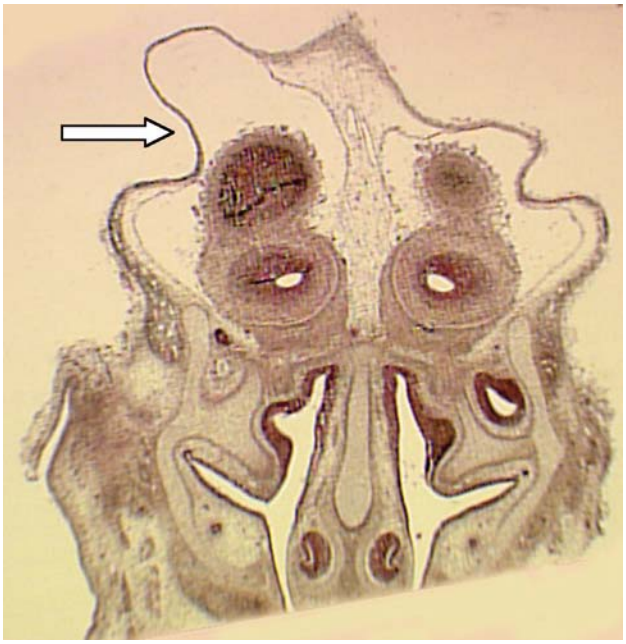
Groups E-F (30 mg kg^{-1} bw 13-cis-RA, GD 9, 10, 11, 12)

None of the 17 foetuses obtained from these two groups exhibited any major external craniofacial malformations. However, Gunston et al. (2005) have reported the presentation of cleft lip and palate in these specimens.

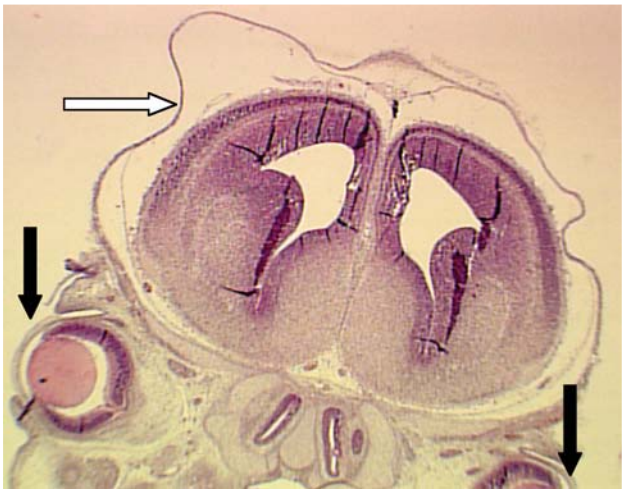
Group G (20 mg kg^{-1} bw acitretine, GD 8, 9, 10, 11)

This specimen presented with bilateral exophthalmos with ablepharia and facial tags. It did not show exencephaly but had a malformed cranial coverage of bone. In the frontal area, the skull was covered by a thin layer of bone bilaterally; while in the midline there was no evidence of bone formation (Figure 6).

Figure 3. Group B (50mg kg^{-1} bw all-trans RA administered GD 9, 10, 11), at GD18.



A - coronal section showing exencephaly as indicated by the white arrow (H & E stain; x 10). B - coronal section showing exencephaly as indicated by the white arrow and bilateral exophthalmos as indicated by the black arrows. (H & E stain; x 10).



A **B**

Discussion

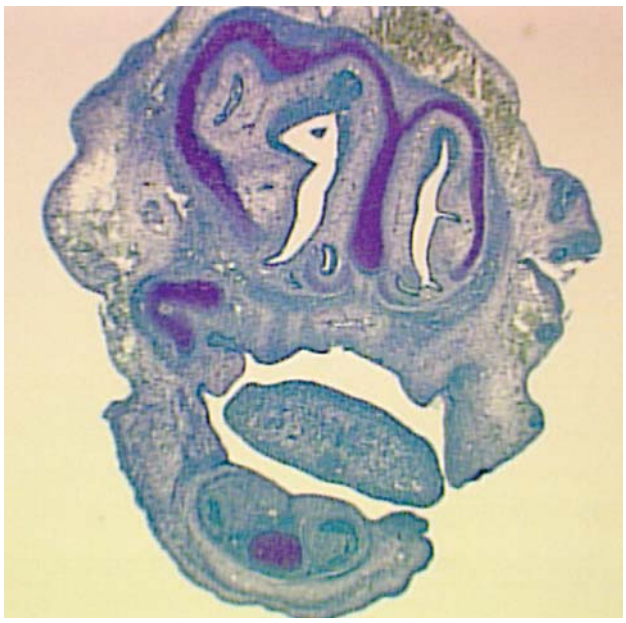
That RA analogues are able to cause teratogenic effects on the developing craniofacial complex of animals and humans has already been reported (Klug et al., 1989; Sulik et al., 1989; Grant et al., 1997; Degitz et al., 1998; Fischer et al., 1999; Emmanouil-Nikoloussi et al., 2000a, 2000b, 2003; Ikemi et al., 2001; Cuervo et al., 2002; Gunston et al., 2005). Here, for the first time, is a description of major craniofacial abnormalities produced with a variety of RAs, employing histological examination.

Despite an extensive literature, it remains unknown exactly how RA analogues exert their teratogenic effects upon the developing foetus (Ross et al., 2000; Emmanouil-Nikoloussi et al., 2003; Gunston et al., 2005). It is however clear that either a deficiency of vitamin A (from which RA is derived) or an excess of RA itself can result in developmental abnormalities (Morriss-Kay, 1993; Zile, 1998; Tzimas and Nau, 2001a, b). It is also evident that RA is essential to the process of normal craniofacial development, as well as having the capacity to disrupt the developmental process (Richman, 1992; Ross et al., 2000). The resulting range of developmental anomalies may then present individually or as constituent parts of a developmental syndrome (Sulik et al., 1989; Gorlin, 1990; Webster and Richie, 1991). Sulik and Dehart (1988) observed that RA exposure in the posterior aspect of the maxillary and mandibular prominences of the mouse embryo yielded malformations similar to those seen in human mandibulofacial dysostosis (Treacher-Collins Syndrome). We have also presented similar findings in this report.

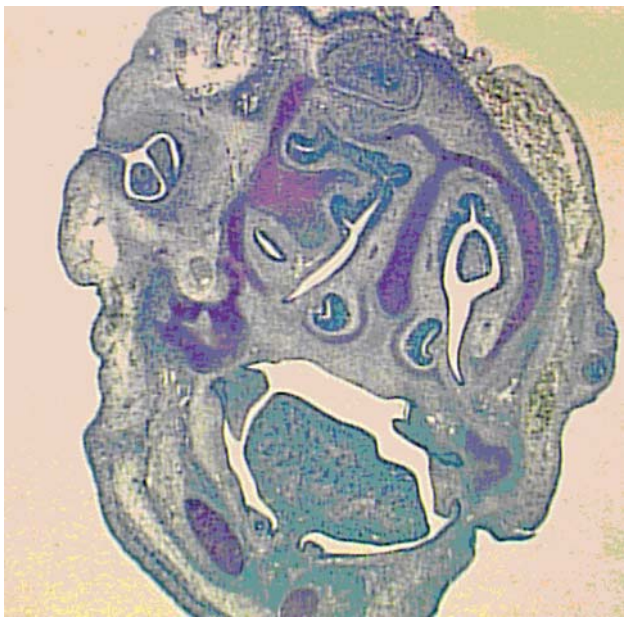
Previous studies have employed a variety of animals, dosage regimens and RA analogues, resulting in a wide spectrum of observed malformations, including anomalies of the cardiovascular, respiratory, uro-genital and nervous systems, in addition to disruption of the craniofacial complex (Morriss-Kay and Sokolova, 1996; Zile, 1998). However, relatively few reports are available that describe specimens exhibiting major craniofacial malformations such as exencephaly, gross craniofacial asymmetry, anophthalmos and exophthalmos, as the result of experimental RA administration (Emmanouil-Nikoloussi et al., 2000c, 2003). The value of this report lies in presenting a collection of specimens that exhibit major external craniofacial malformations as a result of experimental RA administration, and relating the anomalies observed to the form, dosage and timing of foetal exposure to RA.

Our findings describe Wistar rat foetuses that exhibited major craniofacial anomalies similar to those reported by Emmanouil-Nikoloussi et al. (2000c). The narrower range

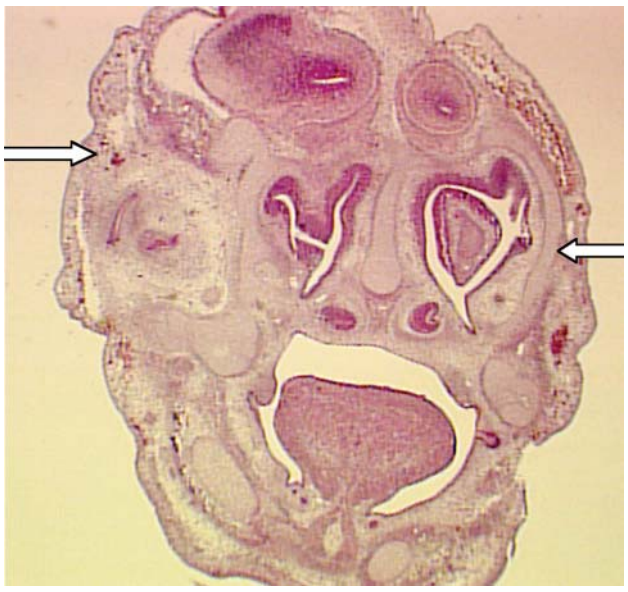
Figure 4. Group C (100mg kg^{-1} bw all-trans RA administered GD 9.5, 10.5), at GD18.



A



B



C

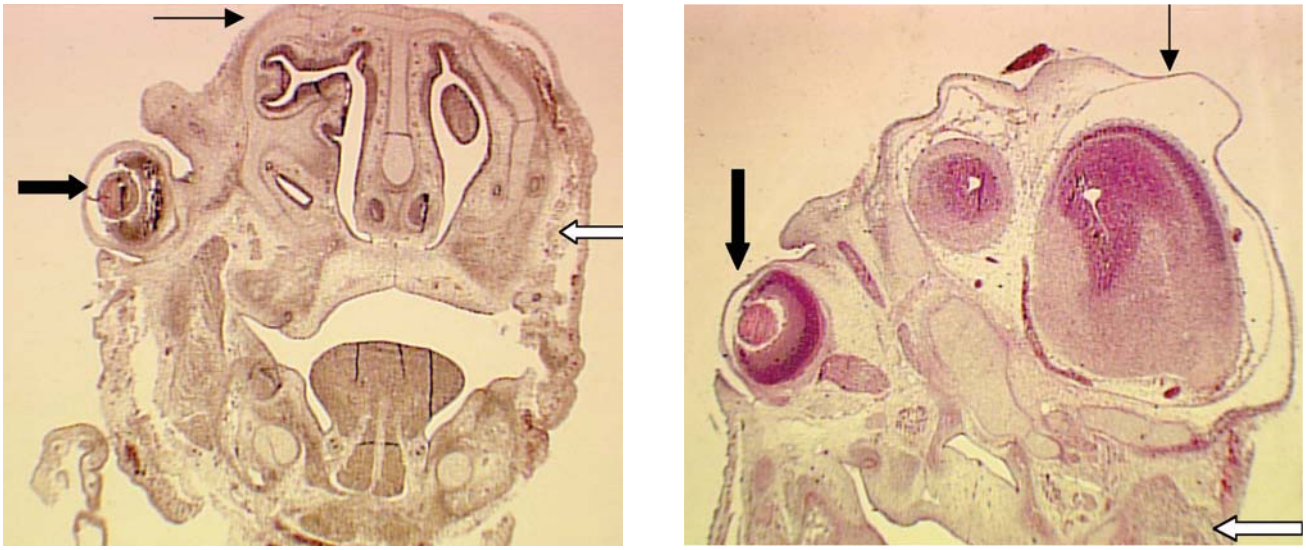
A - coronal section showing gross craniofacial asymmetry (Masson's Trichrome stain; x 10). B - coronal section showing gross craniofacial asymmetry. (Masson's Trichrome stain; x 10). C - coronal section showing gross craniofacial asymmetry and bilateral anophthalmos. The white arrows indicate the approximate areas in which eye development would normally be expected to occur. (H & E stain; x 10).

of anomalies observed in this study reflects the smaller number of specimens obtained with comparison to the work of Emmanouil-Nikoloussi et al. (2000c). However, we are able to report that, in this study, the specimens exhibiting major craniofacial malformations were obtained from a much wider range of treatment regimens.

Firstly, it should be noted that exposure of the developing foetuses to a dosage of 30 mg kg^{-1} bw 13-cis-RA on GD 9, 10, 11, 12 (Groups E and F) did not result in the formation of major craniofacial abnormalities. Gunston et al. (2005) do nevertheless report the incidence of cleft lip and palate in rats treated with an identical experimental regimen. However, at least one specimen from each group exposed to all-trans RA presented with major craniofacial malformations. This accords with the body of literature suggesting that all-trans RA is more teratogenically potent than 13-cis-RA. For example, Klug et al. (1989) suggested that all-trans RA is 3 to 10 times more active in eliciting teratogenic effects than 13-cis-RA.

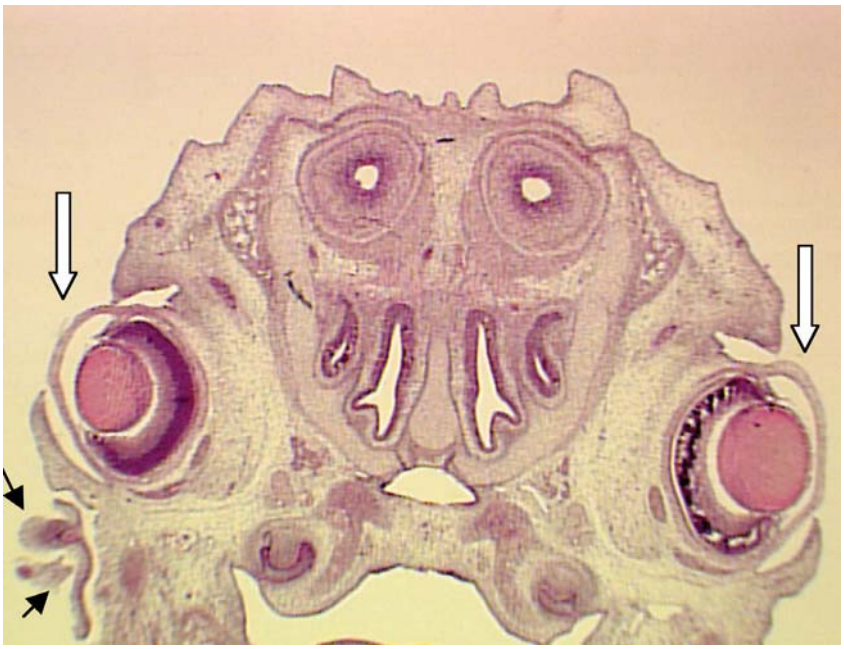
Our results also demonstrate the increased teratogenic effect of exposure to an increased dosage of all-trans RA. Groups A and B were exposed to 30 mg kg^{-1} bw all-trans RA on GD 8, 9, 10, 11 and to 50 mg kg^{-1} bw all-trans RA on GD 9, 10, 11 respectively. The resultant specimens presented very similarly with exencephaly and bilateral exophthalmos. This demonstrates that the relatively small difference in dose, and the administration of the dose to Group A one day earlier than to Group B, had little effect on the extent of the resulting gross craniofacial malformations. However,

Figure 5. Group D (100mg kg^{-1} bw all-trans RA administered GD 8, 9), at GD18.
A - coronal section showing exophthalmos on the right side of the specimen as indicated by the black arrow, anophthalmos on the left side of the specimen as indicated by the white arrow and exencephaly as indicated by the line arrow (H & E stain; x 10). B - coronal section showing exophthalmos on the right side of the specimen as indicated by the black arrow, anophthalmos on the left side of the specimen as indicated by the white arrow and exencephaly as indicated by the line arrow (H & E stain; x 10).



A B

Figure 6. Group G (20mg kg-1 bw acitretine administered GD 8, 9, 10, 11), at GD18.
Coronal section showing bilateral exophthalmos as indicated by the white arrows and facial tags as indicated by the black arrows (H & E stain; x 10).



A

this information must be considered in conjunction with the report of Gunston et al. (2005), in which significant differences in the histological presentation of cleft palate were reported between the two groups.

Upon administration of the larger dose of 100 mg kg^{-1} bw all-trans RA to Group C (GD 9.5, 10.5) and Group D (GD 8, 9), a significantly greater degree of craniofacial malformations was observed. The earlier exposure of the Group D specimen resulted in exencephaly with unilateral anophthalmos and unilateral exophthalmos, while the later exposure of the Group C specimen resulted in gross craniofacial asymmetry with bilateral anophthalmos and facial tags. Emmanouil-Nikoloussi et al. (2003) also observed anophthalmia in exencephalic rat embryos following experimental

administration of all-trans RA, when administered in smaller doses but over a longer period of time than in the present study. These results suggest that the exposure of the foetus to the high dose of RA on GD 9.5, 10.5 had a more powerful teratogenic effect than exposure to the same dose on GD 8, 9. However, although this is a significant observation, further studies are required to confirm this view.

Another RA analogue used in this study was acitretine. This is an aromatic retinoid used in the treatment of severe forms of psoriasis and keratinizing cutaneous disorders. It has extensive side-effects and is known to be a potent teratogen (Guillonneau and Jacqz-Aigrain, 1997; Berbis, 2001). One of the two foetuses obtained from this group presented with bilateral exophthalmos and facial tags. These foetuses were exposed to 20 mg kg^{-1} bw acitretine on GD 8, 9, 10, 11. Therefore, this specimen demonstrates a degree of major craniofacial malformation, even at a very low dosage when administered over this four-day period. This strengthens the evidence for the strong teratogenicity of this compound.

The differential effects of form, dosage and timing of RA administration on embryonic craniofacial development have been the focus of many research studies. The exact relationship between these factors has yet to be resolved and will surely become clearer with the development of future projects. This study demonstrates that it is possible to experimentally induce major craniofacial dysmorphogenesis as a result of exposing developing rat foetuses to particular regimens of RA administration. This will be of value for future studies into, for example, the teratogenic effects of retinoids on the central nervous system. As future studies continue to uncover the precise mechanisms by which RA exerts its effects on the developing tissues at cellular levels, our understanding of the roles of RA in craniofacial morphogenesis will become clearer. Finally, in recent times, RAs (particularly all-trans RA and 13-cis-RA) have been re-introduced therapeutically to treat cancers (including leukaemia and skin cancers) (e.g. Hanson and Leachman, 2001; Robert et al., 2006). Consequently, this treatment could result in congenital abnormalities in pregnant patients and thus studies into the role of RAs in development open new avenues for clinical and experimental investigations.

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