Palatal abnormalities in the developing rat induced by retinoic acid

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SUMMARY

Palatogenesis is a complex developmental process that requires two main events: elevation and then fusion of the palatal shelves. These processes are disrupted by teratogens such as retinoic acid (RA) and genetic defects, resulting in various malformations (including cleft palate). Using histological and immunohistochemical techniques, the effects of different isomers of RA, administered in various concentrations to pregnant rats on different gestational days (GD), were assessed from observations of the state of palatal development on GD 18 in foetuses without exencephaly. Varying degrees of clefting of the palate were observed, from failure of elevation of the palatal shelves to failure of fusion in the midline. This study shows that all-trans-RA is the most teratogenic RA isomer in terms of rat palatal abnormalities. It also supports previous findings that the timing of administration of all-trans-RA is more critical than the concentration, with treatment between GD 10 and 10.5 having the most severe effects. Previous histological studies also suggested that RA is associated with the appearance of ectopic cartilages within the developing palate of foetuses showing exencephaly. In this investigation, immunohistochemical labelling of the foetal material with antibodies that recognise epitopes present in link proteins 1, 2, and 3 (8A4), chondroitin-4-sulphate stubs (2B6), and G1 and chondroitin sulphate attachments (7D1) present in aggrecan (associated with hyaluronan in cartilage) showed no signs of ectopic cartilage formation within the palate at GD18. Internal controls of the cartilages of the nasal septum, vomeronasal cartilage, and Merkel's cartilage labelled intensely and appeared morphologically normal.

Keywords: Palatogenesis – Malformations – Palatal clefts – Retinoic acid – Rat foetuses

INTRODUCTION

Craniofacial abnormalities constitute a significant proportion of congenital malformations (approximately 15 in every 1000 births) and clefts of the palate are one of the most common congenital malformations, accounting for 65 per cent of all congenital craniofacial anomalies (Ferguson, 1981; Schutte and Murray, 1999). It has also been reported that 1 in 500 neonates have some kind of craniofacial cleft (Ferguson, 1981). Clefts of both the palate and lip are more common in males (2:1), although isolated cleft palate is more prevalent in females (2:1), possibly as a result of palatal fusion occurring one week later in females (Sperber, 2001). Cleft palates vary in terms of the degree of severity, the mildest form being cleft of the uvula, which occurs relatively late in development. Submucosal clefting cannot be seen from surface examination, as the mucosa is intact and only the underlying tissues fail to merge in the midline. The most severe forms of cleft palate affect both the hard and soft palate and result in a common oro-nasal cavity. Cleft palate may be associated with cleft lip, although these are inde-

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pendently determined as they have different developmental pathways (e.g. Berkovitz et al., 1992; Sperber, 2001; Meikle, 2002) and both are polygenic multifactorial conditions.

Experimental studies of cleft palate using a variety of animals often involve administration of teratogens to induce clefting. Teratogens known to induce cleft palate include chlorcyclizine (Posner et al., 1967), fluro-2-desoxyuridine (FUDR; Ferguson, 1978b; Singh et al., 1997) and retinoic acid (RA) (e.g. Lotosh, 1968; Sulik et al., 1989; Degitz et al., 1998; Emmanouil-Nikoloussi et al., 2000a, b, 2003). The effect of a teratogen on cleft palate formation depends on the genetic susceptibility of the species (and indeed the particular strain of species), the exact timing of the administration of the teratogen (as most teratogens only produce their effects at certain stages [or critical periods] of development) and the drug dosage (e.g. Singh, 1991; Ikemi et al., 2001). Cleft palate can be caused at different stages of palatogenesis as a result of incomplete growth, elevation or fusion of the palatal shelves (Ferguson, 1981; Abbott et al., 1989; Abbott and Birnbaum, 1990) although the exact mechanism(s) remains unknown. Both a deficiency and an excess of RA leads to complications and causes harmful and widespread effects. RAs disrupt the critical processes involved in craniofacial development, including neural crest cell migration (Lee et al., 1995), ECM synthesis (Lorente and Miller, 1978) and cell proliferation and differentiation, possibly by interaction with growth factors (Kochhar, 1968; Nanda, 1971; Abbott and Pratt, 1988; Cuervo et al., 2002). RA can induce cleft palate in the foetus at levels that are not normally maternally toxic, not embryo-lethal and do not increase the re-absorption rates (Abbott and Birnbaum, 1990). Previous studies concerning the effects of all-trans-RA on the craniofacial development of the rat foetus have been conducted by Emmanouil-Nikoloussi et al. (2000a; b; 2003). They reported that following the administration of all trans-RA between gestational days 8 to 12, and based upon observations on gestational day 21, this RA was both time and dosage dependent. This finding supports the hypothesis that all-trans-RA disturbs the growth and differentiation necessary for normal craniofacial development. However, the mechanism of action and interference of the drug still remains unclear (Emmanouil-Nikoloussi et al., 2000a, b). Emmanouil-Nikoloussi et al. (2000b) also reported that treatment with RA results in the formation of aberrant cartilages within the developing rat palate.

The aims of this study were three-fold. First, to investigate the dosage and developmental stage at which different RA analogues have teratogenic effects on palatal development in the Wistar rat. Second, to assess the degree of palatal clefting and the characteristics of the ectopic cartilages appearing after RA exposure. Third, and in comparison with the earlier research of Emmanouil-Nikoloussi et al. (2000a, b), to observe the extent of palatal malformations occurring earlier than on GD 21.

Materials and Methods

Twenty-two *"prima gravida"* female rats (Wistar), aged 3 months, and weighing 200-250g, were mated overnight. GD 0 was indicated by the presence of a vaginal plug the next morning. The rats were then maintained in isolation at 20ºC ± 2ºC with a 12 hour light-dark cycle. They were given food and water *"ad libitum"* and their weights were recorded daily.

Corn oil suspensions of RA were prepared in dark-light conditions and the solutions were sonicated and vortexed to obtain a uniform suspension and stored in darkened glass vials. The dosages were calculated per kg body weight of the pregnant rats. The rats were grouped according to the RA analogue used, dosage and the day of treatment (Table 1), and they were fed the RA in corn oil preparation via gastric intubation

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

Table 1. Protocol for RA administration to experimental animals

under mild ether sedation. The rats were closely observed for weight loss, hair loss, hemopthalmus (blood filled eyes), and vaginal bleeding (indicative of miscarriage) to ensure survival.
Pregnant rats were sacrificed by cervical dislocation at GD 18. A perpendicular laparotomy was performed and the uterine horns were fully exposed, opened and the foetuses were removed. Foetal membranes were removed and foetuses were washed in phosphate buffered saline (PBS; 10mM phosphate, 2.7mM KCl, 137 mM NaCl, pH 7.4). Foetal heads were then microdissected under a stereomicroscope and fixed in 10% neutral formalin (phosphate buffered).
All of the above procedures were carried out at the University of Thessaloniki, Greece under licensing rules according to the Helsinki Guidance for Animal Practice.
The foetal heads were subsequently paraffin wax-embedded and serial frontal sections were cut at 7µm using an LKB Historange rotary microtome and mounted onto glass microscope slides coated with glycerine and albumen to aid adhesion, and 'Histobond' slides for immunohistochemical studies. Histological sections were deparaffinized in xylene and rehydrated through a graded alcohol series. They were then stained using Haematoxylin and Eosin, Toludine Blue or Masson's trichrome according to standard histological procedures. Sections mounted under cover glasses using DPX solution were viewed with a Leica Leitz DM RB microscope. Micrographs were taken using a digital camera and Image Grabber PCI software.
Sections for immunohistochemical investigations were deparaffinized in xylene, rehydrated through a graded series of alcohols, and washed (3x 5min, PBS). The sections on each slide were outlined in DAKO pen (DAKO laboratories), which forms a waterproof ring around the sections, allowing localised application of the reagents. The sections were then washed and equilibrated in 100mM Tris-acetate at pH 6.5, containing 4mM 1,10-phenanthroline, for 5 minutes before being incubated for 2 hours at 37ºC with Chondroitinase ABC (Sigma; $0.5Uml^{-1}$), keratanase I (Seikagaku; $0.5Uml^{-1}$) and keratanase II (Seikagaku; 0.005Uml-1; all proteinase-free preparations) prepared in 100mM Tris-acetate (pH 6.5) containing 4mM 1,10-phenanthroline. Sections were washed (3 x PBS for 5 minutes) and incubated for 20 minutes in 1.5% horse serum (in PBS-Tween [PBS-T] containing 4mM 1,10-phenanthroline) before blotting away the excess serum. Primary antibodies, diluted in PBS-T (containing 4mM 1,10-phenanthroline), were applied to the sections (monoclonal antibody 2B6, 1:1600; Caterson et al., 1985b; 7-D-1, 1:2000; 8A4, 1:1000; Caterson et al., 1985a) and incubated at 4 ºC overnight (for approximately 15 hours). Sections were washed (2 x PBS for 5 minutes) and biotinylated secondary antibody applied (60 minutes; horse anti-mouse IgG; Vector laboratories, UK; in PBS-T). After washing (3x 5min PBS), avidin-biotin complex (ABC) reagent (Vector laboratories, UK) in PBS-T was applied (60 minutes). Sections were then incubated with peroxidase substrate solution (DAB peroxidase visualisation kit; Vector laboratories, UK) for 4 minutes before being washed with water and counterstained with haematoxylin (10 seconds). Sections were subsequently dehydrated through a series of alcohols, placed into xylene and mounted with cover glasses using DPX mountant. Sections were viewed using a Leica Leitz DM RB microscope and micrographs were taken using Image Grabber software. Each immunohistochemistry run included a negative control where the primary antibody was omitted and the sections were incubated with PBS solution to ensure specific binding was seen. The secondary antibody was then included to show lack of non-specific binding. The auto-fluorescence of the material was also demonstrated by omitting both the primary and secondary antibodies. The labelling of the nasal septum cartilage and Merkel's cartilage were used as internal positive controls.

RESULTS

Administration of isomers of RA in different doses on different gestational days affected both the gross morphology and the histological appearance of the developing head and palate (Figures 1 to 9 and Table 2). All results were assessed in comparison with the untreated control as it was the same age as the treated material (GD 18). There were no differences between the treated and untreated controls that were not accountable to the difference in age. Three untreated control heads were analysed: all were found to have developed normally. The control heads were assessed to be normal by comparisons with Ferguson's (1978a) report on the development of the rat palate and Kaufman's "Atlas of Mouse Development" (1999). Most litters included teratomas and absorptions as well as foetuses (Table 2). Unless otherwise stated, all observations are bilateral and are from anterior to posterior regions of the palate.

Controls

No abnormalities of the control heads were noted from both gross and histological examinations (Figure 1). Histologically, the palatal shelves had fused (and with the nasal septum) and consequently the oral and nasal cavities were separated (Figure 1c). Posteriorly, the sphenoid cartilage in the region of the presumptive soft palate was bilaterally symmetrical (Figure 1f). These observations were the same for both the untreated specimens and the corn oil-treated control.

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

Group A (30 mg kg^{-1} bw All trans-RA, GD 8, 9, 10, 11)
A cleft lip was apparent from macroscopic observation (Figure 2b). The palatal shelves were in the vertical position (resulting in a bilateral cleft) and the nasal septum was unattached inferiorly (Figure 2c). Further posteriorly, the nasal septum had fused with the lateral nasal walls and separated the nasal and oral cavities, although the palatal shelves remained vertical (Figure 2d). The palatal shelves were vertically orientated throughout the presumptive soft palate. The sphenoid cartilage was regular in shape (Figure 2e). No Common Nasal Passage (CNP) was formed throughout, due to the failure of palatal reorientation.

Group B (50mg kg^{-1} bw All-trans-RA, GD 9, 10, 11)
Macroscopic observations demonstrated the presence of a cleft lip (Figure 3b). Histologically, in the anterior region of the palate, the palatal shelves had reoriented and the nasal septum had orientated between the palatal shelves. The left palatal shelf had formed an epithelial "bridge" with the nasal septum (Figure 3c). Posteriorly, the nasal septum and the lateral nasal wall on the right had fused (Figure 3e) and more posteriorly the CNP had formed (Figure 3f,g). Remains of the Midline Epithelial Seam (MES) were present (Figure 3e,f). The sphenoid cartilage was not bilaterally symmetrical (Figure 3g).

Group C (100mg kg^{-1} bw All trans-RA, GD 9.5, 10.5)
A cleft lip was apparent from macroscopic observations (Figure 4b). Histologically, the right palatal shelf appeared short and vertical in orientation and the left palatal shelf had failed to develop. Ingrowths were present on the inferior epithelium of the nasal septum (Figure 4c). More posteriorly, the epithelium on the left side had invaginated to form an epithelial "bridge" from the Jacobson's organ into the nasal cavity (Figure 4d). It appeared that the nasal septum had fused with the lateral nasal walls in the region of the vomeronasal cartilages and Jacobson's organs, where in normal development it is fused with the palatal shelves. In this region, both palatal shelves were present as short vertical outgrowths (Figure 4d). Posteriorly, the nasal septum was inferiorly unattached. No CNP was formed in any region of the palate. In the presumptive soft palate, the right palatal shelf had grown horizontally towards the midline (Figure 4e) but the left palatal shelf was absent.

Group D (100mg kg^{-1} bw All trans-RA, GD 8, 9)
Macroscopic observation revealed a cleft lip (figure 5b). Histologically, the nasal septum was between the palatal shelves and the left palatal shelf had fused with it. The right palatal shelf was unfused, thus forming a unilateral cleft (Figure 5c). More posteriorly, the palatal shelves had fused and the nasal septum was unattached inferiorly (Figure 5d). Further posteriorly (Figure 5e), the nasal septum fused with the lateral nasal walls to form the CNP that extended into the presumptive soft palate. The sphenoid cartilage was not bilaterally symmetrical (Figure 5f). The MES was still partially present throughout the palate (Figure 5d-f).

Group E (30 mg kg^{-1} bw 13 cis-RA, GD 9, 10, 11, 12)
Macroscopic observations showed a slight cleft of the upper lip (Figure 6b). Anteriorly, the nasal septum intervened with the palatal shelves and the right palatal shelf had fused with it. The left palatal shelf and the nasal septum were linked by an epithelial "bridge" (Figure 6c). Posterior to this, the lateral nasal wall outgrowth had begun to fuse with the nasal septum via an epithelial

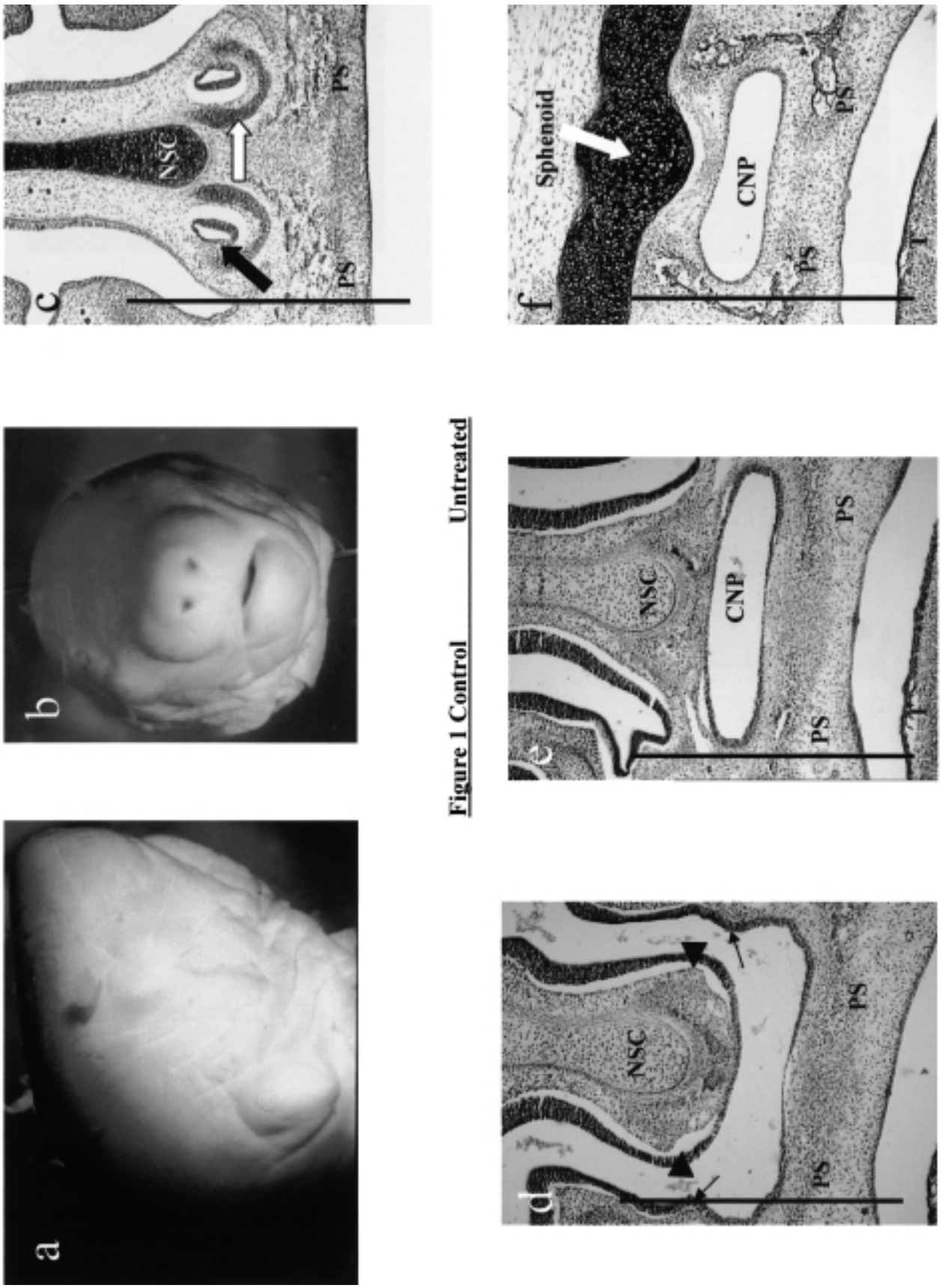

Fig. 1.- Control (Untreated GD18). a) Macroscopic lateral view of a foetal control head. x 4. b) Macroscopic frontal view of a foetal head. Note the fused lip (lack of cleft lip) and symmetrical formation of the head. x 4. c) Coronal section of the anterior region of the presumptive hard palate showing fusion of the palatal shelves (PS) with each other and with the nasal septum (NSC). Symmetrical vomeronasal cartilages (white arrow) and Jacobson's organs (back arrow) can be seen. (Toludine Blue). d) Coronal section of the mid region of the presumptive hard palate. The nasal septum (NSC) is unattached inferiorly. Note the lateral outgrowths of the nasal septum (arrow-heads) and the medial outgrowths of the lateral nasal walls (arrows). (PS = palatal shelves). (H & E). e) Coronal section of the posterior region of the presumptive hard palate. The lateral nasal walls have fused to form the Common Nasal Passage (CNP) with the palatal shelves (PS) forming its inferior border. Note the ovoid appearance of the CNP and the even epithelia. (Masson's Trichrome). f) Coronal section of the presumptive soft palate. The sphenoid cartilage is bilaterally symmetrical and the palatal shelves (PS) are fused. The CNP is ovoid in appearance. (Toludine Blue). Scale bars for c) to f) = 1mm.

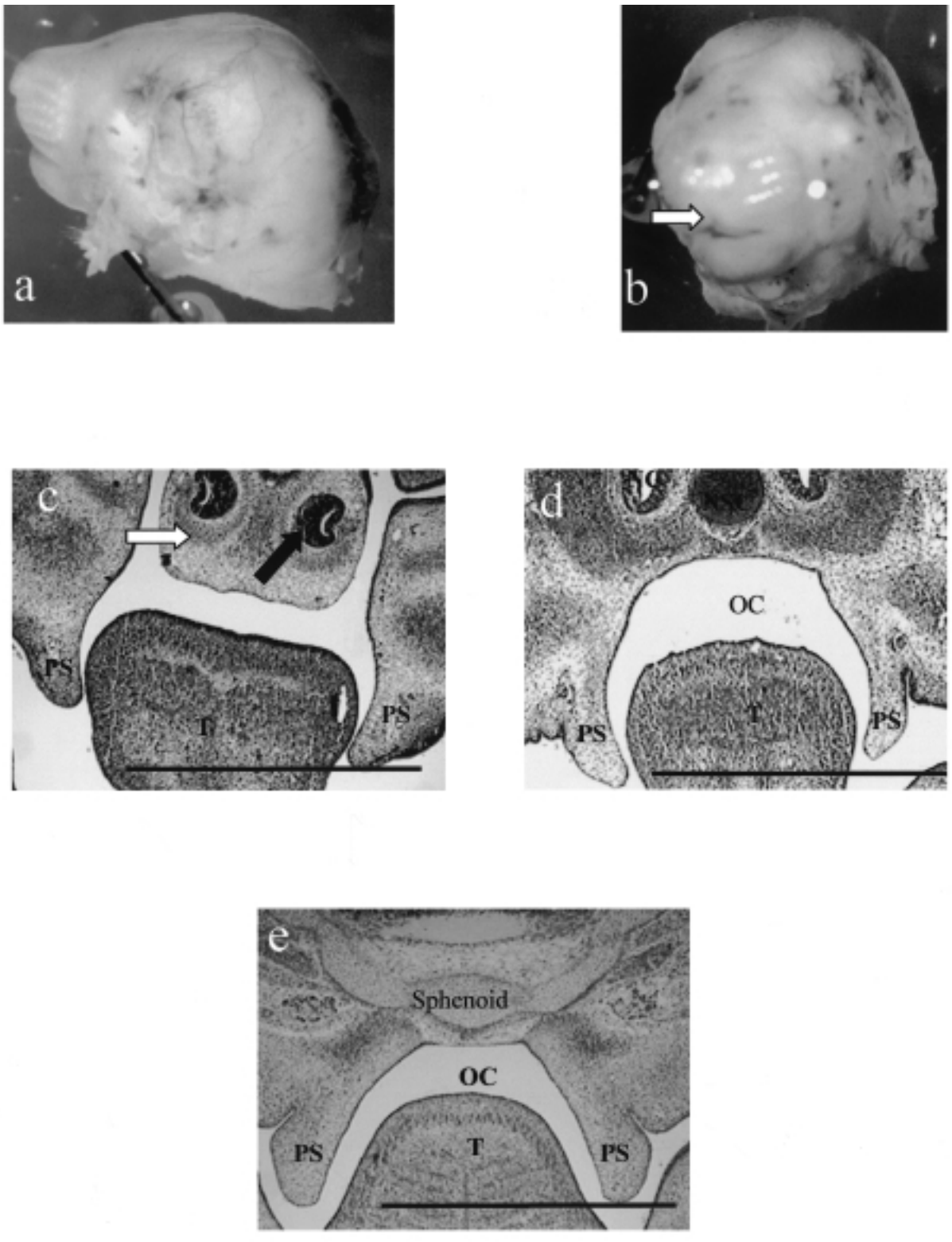

Figure 2 Group A 30mg kg^{-1} bw All trans-RA, GD 8, 9, 10, 11

Fig. 2.- Group A (30 mg kg^{-1} bw All trans-RA, GD 8, 9, 10, 11). a) Macroscopic lateral view of a foetal head. x 4. b) Macroscopic frontal view of a foetal head. Arrow indicates cleft lip. x 4. c) Coronal section of the anterior region of the presumptive hard palate. The palatal shelves (PS) are in the vertical position lateral to the tongue (T). The nasal septum is unattached inferiorly. The white arrow indicates vomeronasal cartilage and the black arrow indicates Jacobson's organs. (Masson's Trichrome). d) Coronal section of more posterior region of the presumptive hard palate. The palatal shelves (PS) are still vertically orientated lateral to the tongue (T). The nasal septum (NSC) has fused laterally with the lateral nasal walls, separating the oral (OC) and nasal cavities (NC). (Toludine Blue). e) Coronal section of more posterior section of the presumptive soft palate. Note the palatal shelves (PS) are still in vertical orientation and that the sphenoid is bilaterally symmetrical. (OC = oral cavity; T = tongue). (H & E). Scale bars for c) to e) = 1mm.

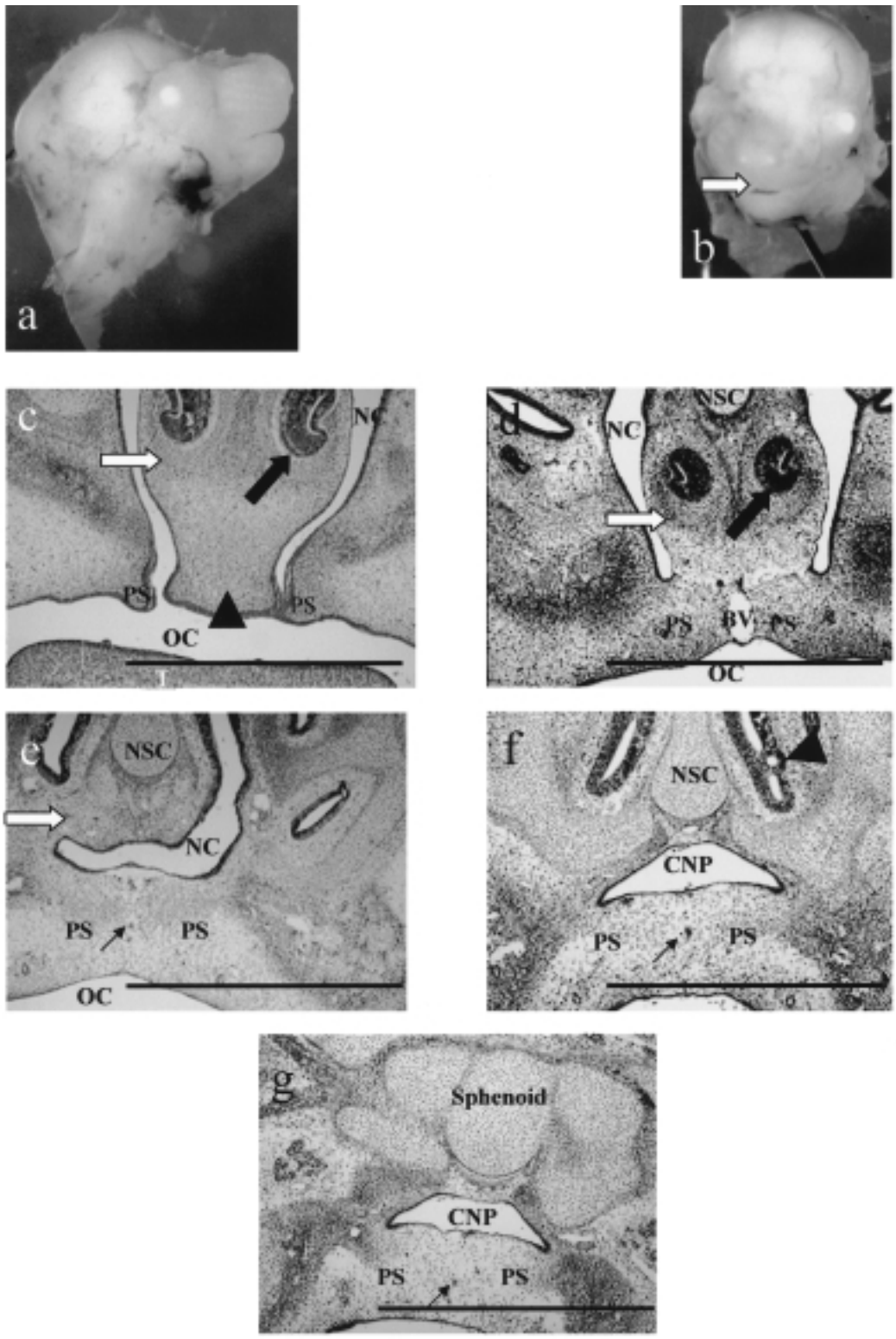

Figure 3 Group B 50mg kg^{-1} bw All trans-RA, GD 9, 10, 11

Fig. 3.- Group B (50mg kg $^{-1}$ bw All-trans-RA, GD 9, 10, 11). a) Macroscopic lateral view of the foetal head. x 4. b) Macroscopic frontal view of the foetal head. Arrow indicates cleft lip. x 4. c) Coronal section of the anterior region of the presumptive hard palate. The white arrow indicates vomeronasal cartilage and black arrow indicates Jacobson's organs. Note the short palatal shelves (PS), inferior extension of the nasal septum and fusion via an epithelial bridge of the right palatal shelf and the inferior region of nasal septum. The arrowhead marks the inferior extension of the nasal septum. (T = tongue; OC = oral cavity). (H & E). d) Coronal section of a more posterior region of the anterior presumptive hard palate. Note the blood vessel (BV) formation between the palatal shelves. The palatal shelves (PS) and the nasal septum (NSC) have fused, separating the oral and nasal cavities (OC and NC). (Masson's Trichrome). e) Coronal section of a more posterior region of the presumptive hard palate. The nasal septum (NSC) is fused laterally with the lateral nasal wall (white arrow). The palatal shelves (PS) are fused. Note remains of the Midline Epithelial Seam (MES) (arrow). (OC = oral cavity; NC = nasal cavity). (H & E). f) Coronal section of a more posterior section of the presumptive hard palate. Note fusion of the nasal septum (NSC) with the lateral nasal walls and formation of the CNP. Arrowhead shows epithelial fusion of the lateral nasal wall with the nasal septum. Note remains of the MES (arrow) (PS = palatal shelves). (H & E). g) Coronal section of the presumptive soft palate. The CNP is present. Note that the sphenoid cartilage is not bilaterally symmetrical. Note also remains of the MES (arrow). (PS = palatal shelves). (Masson's Trichrome). Scale bars for c) to g) = 1 mm.

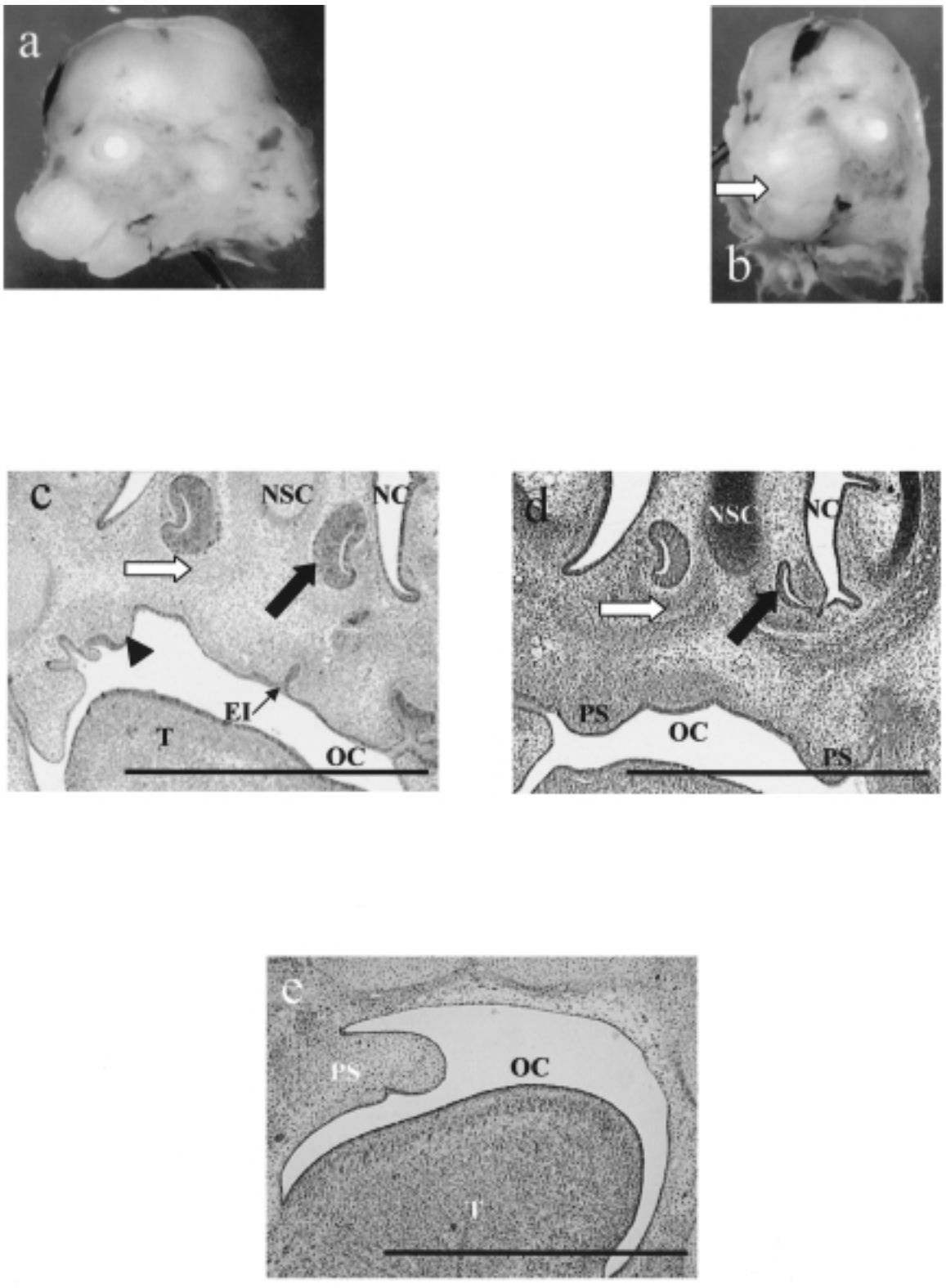

Figure 4 Group C 100mg kg^{-1} bw All trans-RA, GD 9.5 10.5

Fig. 4.- Group C (100 mg kg^{-1} bw All rans-RA, GD 9.5, 10.5) a) Macroscopic lateral view of the foetal head. x 4. b) Macroscopic frontal view of the foetal head. Arrow indicates cleft lip. x 4. c) Coronal section of the anterior presumptive hard palate. The nasal septum (NSC) is fused laterally with the lateral nasal walls. Note the ingrowth of the inferior epithelial border (EI). The arrowhead indicates formation of the left palatal shelf, which is small and underdeveloped. The right palatal shelf is absent. The white arrow indicates the vomeronasal cartilage and the black arrow indicates the Jacobson's organs. (T = tongue; NC = nasal cavity; OC = oral cavity). (H & E). d) Coronal section of a slightly more posterior section of the presumptive hard palate. The nasal septum (NSC) is fused with the lateral nasal wall. The palatal shelves (PS) are short and vertical in orientation. The white arrow indicates the vomeronasal cartilage and the black arrow indicates the Jacobson's organs. (NC = nasal cavity; OC = oral cavity). (Toludine Blue). e) Coronal section of the presumptive soft palate. Note the horizontal orientation of the right palatal shelf (PS) and the absence of the left palatal shelf resulting in no CNP. (OC = oral cavity; T = tongue). (Masson's Trichrome). Scale bars for c) to e) = 1 mm.

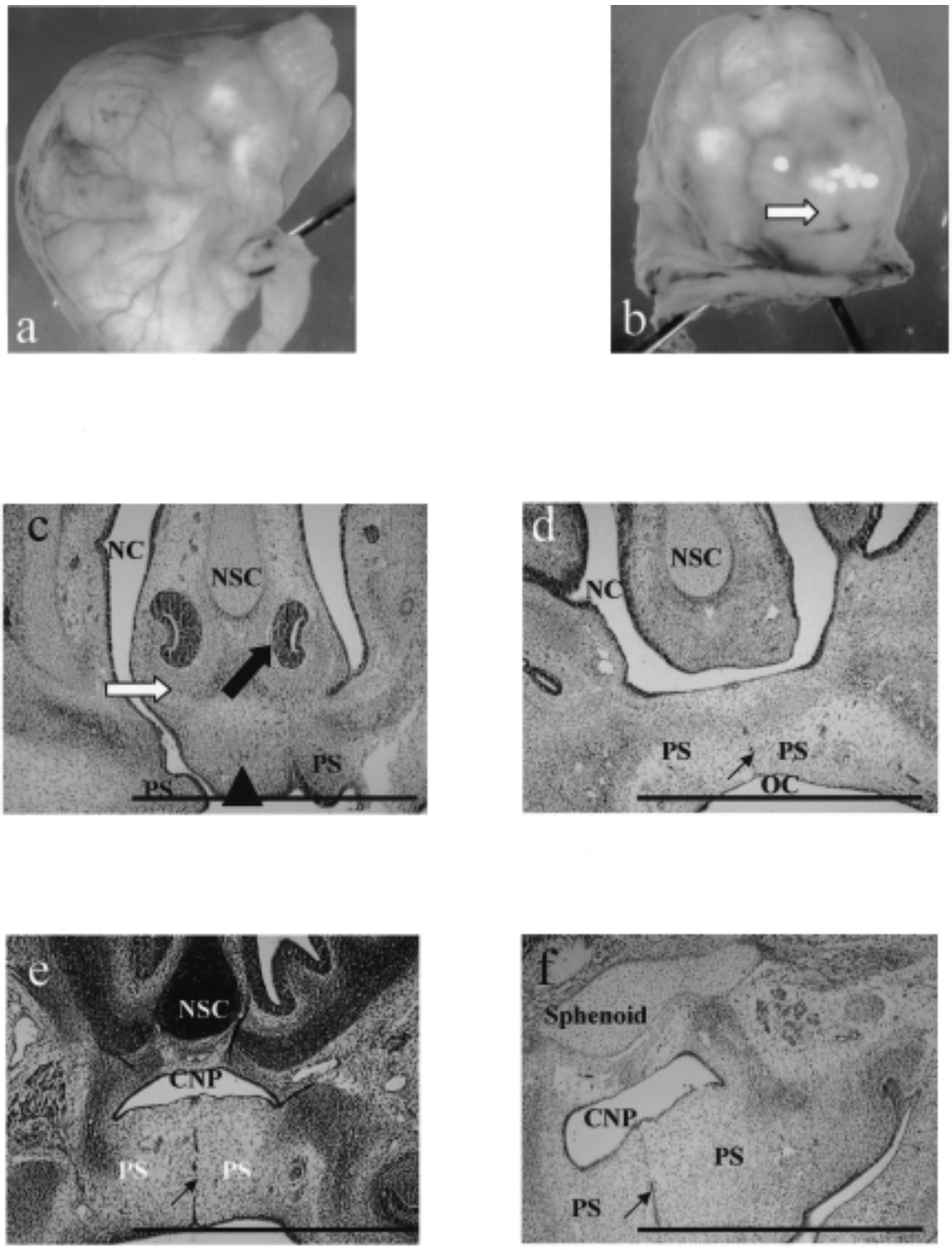

Figure 5 Group D 100mg kg^{-1} bw All trans-RA, GD 8, 9

Fig. 5.- Group D (100 mg kg $^{-1}$ bw All trans-RA, GD 8, 9) a) Macroscopic lateral view of the foetal head. x 4. b) Macroscopic frontal view of the foetal head. Arrow indicates cleft lip. x 4. c) Coronal section of anterior region of the presumptive hard palate. Note that the left palatal shelf (PS) has fused with the nasal septum (arrowhead) but there is no fusion present on the right. The white arrow indicates the vomeronasal cartilage and the black arrow indicates the Jacobson's organs. (NC = nasal cavity; NSC = nasal septum cartilage.) (H & E). d) Coronal section of a more posterior section through the presumptive hard palate. The nasal septum (NSC) is unattached inferiorly. The palatal shelves (PS) are fused. Note remains of the Midline Epithelail Seam (MES) (arrow). (OC = oral cavity; NC = Nasal Cavity.) (Masson's Trichrome). e) Coronal section of the posterior region of the presumptive hard palate. The Common Nasal Passage (CNP) is formed due to the lateral expansion of the nasal septum (NSC) and the medial growth of the lateral nasal walls. Note remains of the MES (arrow). (PS = palatal shelves). (Toludine Blue). f) Coronal section of the presumptive soft palate. The sphenoid cartilage is irregular in shape and the MES (arrow) is present. (PS = palatal shelves; CNP = Common Nasal Passage). (Masson's Trichrome). Scale bars for c) to f) = 1 mm.

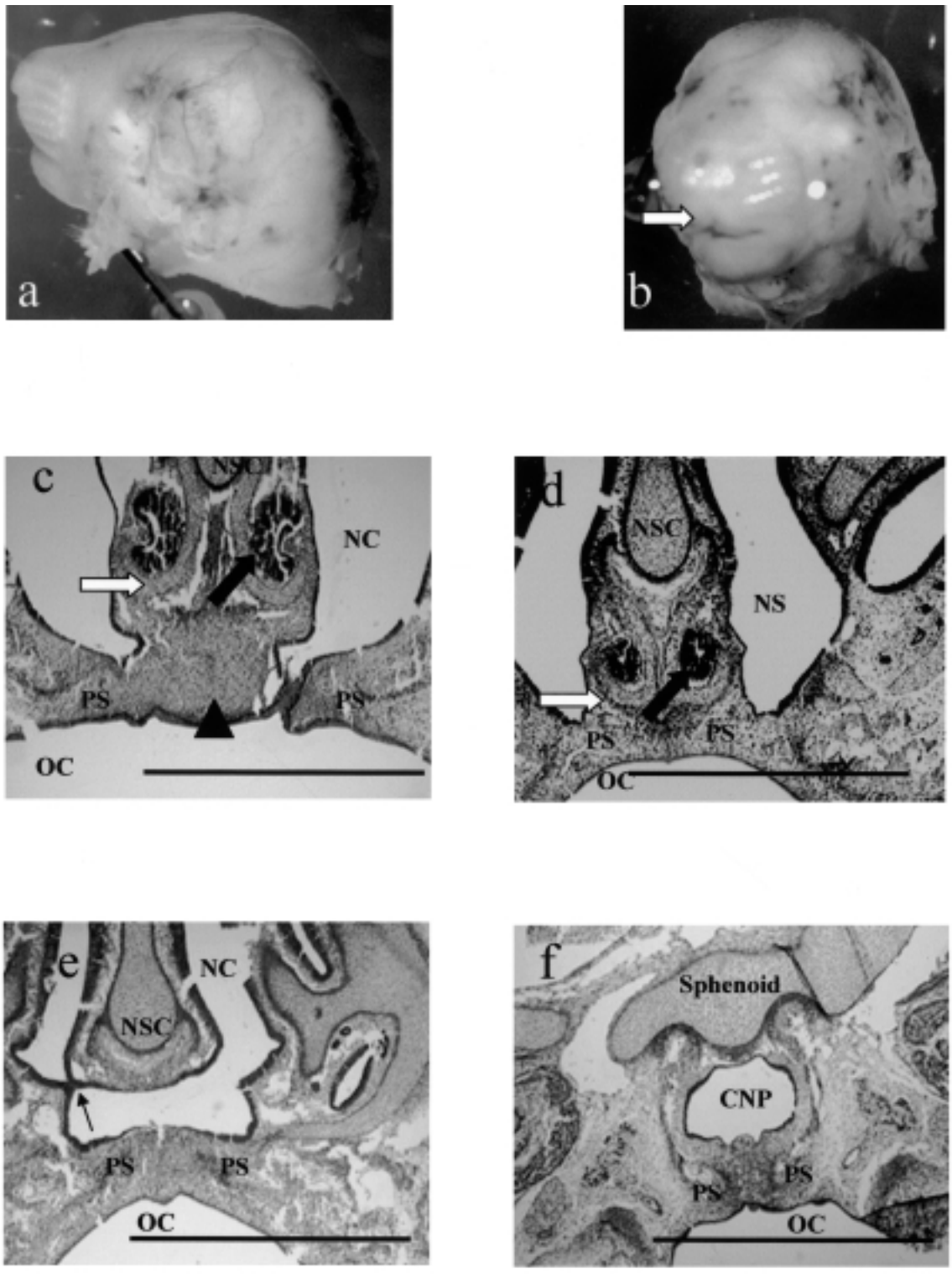

Figure 6 Group E 30mg kg^{-1} bw 13 cis-RA, GD 10, 11, 12

Fig. 6- Group E (30 mg kg $^{-1}$ bw 13 cis-RA, GD 9, 10, 11, 12) a) Macroscopic lateral view of the foetal head. x 4. b) Macroscopic frontal view of foetal head. Arrow indicates cleft lip. x 4. c) Coronal section of the anterior region of the presumptive hard palate. Note the nasal septum (arrow-head) is between the palatal shelves (PS). The right palatal shelf is fused with the nasal septum. The left palatal shelf is fused to the nasal septum by an epithelium "bridge". The white arrow indicates the vomeronasal cartilage and the black arrow indicates the Jacobson's organs. (NSC = nasal septum cartilage; OC = oral cavity; NC = nasal cavity). (H & E). d) Coronal section of a more posterior section through the presumptive hard palate. The palatal shelves (PS) are fused with each other and with the nasal septum (NSC). The white arrow indicates the vomeronasal cartilage and the black arrow indicates the Jacobson's organs. (OC = oral cavity; NC = nasal cavity). (H & E). e) Coronal section of a more posterior region of the presumptive hard palate. Note the palatal shelves (PS) are fused and the nasal septum (NSC) is unattached to the palatal shelves. The nasal septum is attached to the right lateral nasal wall via an epithelial "bridge" (arrow). (OC = oral cavity; NC = nasal cavity). (H & E). f) Coronal section of presumptive soft palate with a CNP and an irregular sphenoid cartilage. (PS=palatal shelves; OC=oral cavity) (H & E). Scale bars for c) to f) = 1 mm.

"bridge" (Figure 6e), resulting in the formation of the CNP. The palatal shelves had fused in the presumptive soft palate but the superior and inferior epithelia were convoluted. The sphenoid cartilage was not bilaterally symmetrical (Figure 6f).

Group F (30 mg kg^{-1} bw 13 cis-RA, GD 9, 10, 11, 12)
A cleft lip was seen upon macroscopic observation (Figure 7b). Microscopic observations revealed that the palatal shelves and nasal septum had fused, separating the oral and nasal cavities (Figure 7c). Posteriorly, the nasal septum had separated from the palatal shelves (Figure 7d) and, further behind, had extended to join with the lateral nasal walls to form the CNP, which was slightly irregular in shape throughout with convolutions of the superior epithelia of the palatal shelves (Figure 7e). The sphenoid cartilage was bilaterally symmetrical (Figure 7f).

Group G (20 mg kg^{-1} bw Acitretine, GD 8, 9, 10, 11)
A cleft lip was apparent from macroscopic observations (Figure 8b). Histologically, the nasal septum had extended between the palatal shelves, which had started to fuse with the superior region of the left palatal shelf. The right palatal shelf and the nasal septum were fused but an epithelial seam remained. The palatal shelves had epithelial and mesenchymal extensions inferomedially into the oral cavity (Figure 8c). Posteriorly, the nasal septum and the lateral nasal wall formed the CNP (Figure 8d, e). Within the region of the presumptive soft palate, the CNP and the sphenoid cartilage were not bilaterally symmetrical (Figure 8f).

Immunohistochemistry
Immunohistochemical analysis of the palate revealed no cartilage specific molecules within the palate of control or experimental animals, regardless of treatment (Figure 9a-f). No positive labelling was present in the negative control sections, demonstrating the lack of non-specific binding and tissue autofluorescence (Figure 9g, h).

Discussion

There have been many reports describing the various craniofacial malformations induced by RA (Lotosh, 1968; Sulik et al., 1989; Abbott et al., 1989; Abbott and Birnbaum, 1990; Abbott et al., 1990; Morriss-Kay, 1993; Jacobsson and Granstrom, 1997; Degitz et al., 1998; Makori et al., 1998; Fischer et al., 1999; Emmanouil-Nikoloussi, 2000a, b, 2003; Suwa et al., 2001; Ikemi et al., 2001; Cuervo et al., 2002). To date, however, the mechanisms of teratogenesis are still poorly understood, despite several decades of intense research. It is well known that, in addition to their roles in controlling many normal developmental processes, retinoids have teratogenic effects in experimental animal models. Indeed, both excess and deficiency of RAs are teratogenic. Embryological developmental studies support the theory that the role of RA is mediated by different retinoid receptors; using retinoid ligands, their role was determined: RAR alpha-ligand induced the most varied defects, such as severe ear, mandible and limb malformations; RAR beta-ligand induced defects in the urinary system and liver; RAR gamma-ligand elicited ossification defects (Elmazar et al., 1996). Kraft et al. (1989) compared the teratogenic dosage of the trans- and cis-forms of RA and found that the cis-forms (and their 4-oxo derivative) were less teratogenic.
Furthermore, as indicated by Emmanouil-Nikoloussi et al. (2000a, b) and Emmanouil-Nikoloussi et al. (2003), investigations are now needed to address issues related to the timing of treatment and to the dosage of RA. The main aim of the present study was consequently to assess the effects of a variety of RA treatments on the development of the rat palate. In addition, using histological and immunohistochemical techniques, we tested the hypothesis that RA causes ectopic cartilage formation within the developing rat palate.
For all the experiments reported in the present paper, RA treatment was commenced before the formation of the palatal shelves, which in the rat develop between GD14 and 15 (Ferguson, 1988). In all the foetal heads investigated, the nasal septum had detached from the palatal shelves to allow the nasal flanges to fuse with the medial ingrowths of the lateral nasal walls. This is in accordance with observations of palatal development within the control specimens. Treatment with RA was seen not to cause disruptions to the development of the nasal septum. However, the common nasal passage formed only in the heads of those foetuses where palatal shelf reorientation had occurred. In these heads, the nasal septum was present between the palatal shelves and thus prevented their fusion in the midline. Consequently, the palatal shelves appeared short in these specimens. It is possible that the effect of RA treatment on the anterior region of the presumptive hard palate in these specimens might have prevented adequate enlargement of the palatal shelves to allow fusion to occur. The presence of the nasal septum allowed partial separation of the oral and nasal cavities by fusion of the nasal septum and the palatal shelves. More posteriorly, where the nasal septum undercuts and separates from the palatal shelves, the palatal shelves were close enough to allow fusion. The palatal shelves failed to reorientate into a horizontal po-

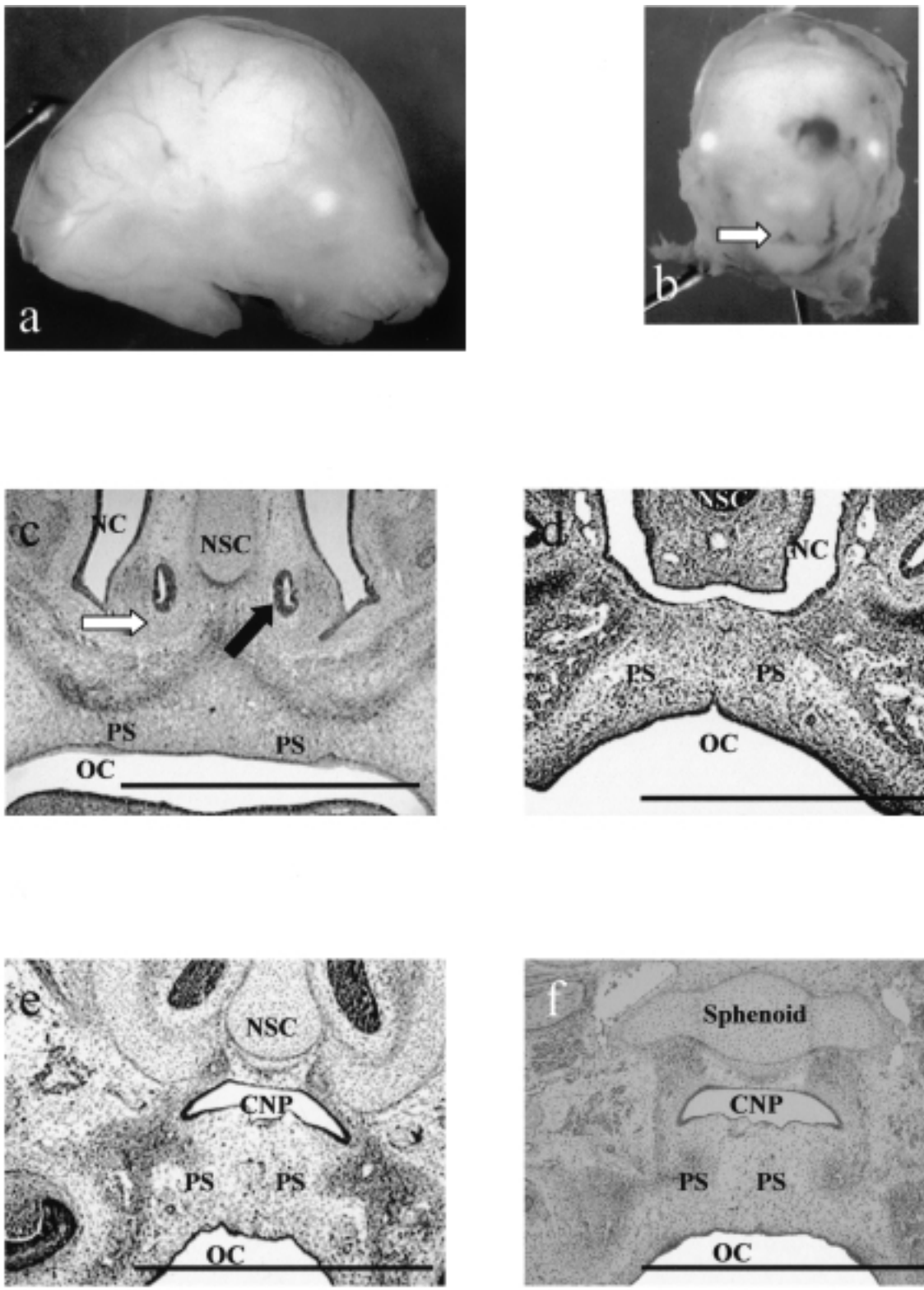

Figure 7 Group F 30mg kg^{-1} bw 13 cis-RA, GD 9, 10, 11, 12

Fig. 7.- Group F (30 mg kg $^{-1}$ bw 13 cis-RA, GD 9, 10, 11, 12). a) Macroscopic lateral view of the foetal head. x 4. b) Macroscopic frontal view of the foetal head. Arrow indicates cleft lip. x 4. c) Coronal section of the anterior region of the presumptive hard palate. The palatal shelves (PS) are fused with each other and with the nasal septum (NSC) separating the oral and nasal cavities (OC and NC). The white arrow indicates the vomeronasal cartilage and the black arrow indicates the Jacobson's organs. (H & E). d) Coronal section of a more posterior section of the presumptive hard palate. The nasal septum (NSC) is unattached inferiorly. The palatal shelves (PS) are fused. (NC = nasal cavity; OC = oral cavity). (Toludine Blue). e) Coronal section of the posterior region of the presumptive hard palate. The palatal shelves (PS) are fused and the CNP is formed. Note the convoluted inferior border of the CNP and the palatal shelves. (NSC = nasal septal cartilage; OC = oral cavity). (H & E). f) Coronal section of the presumptive soft palate. The CNP is present and has a convoluted inferior border. The sphenoid cartilage is bilaterally symmetrical. (OC = oral cavity; PS = palatal shelves). (Masson's Trichrome). Scale bars for c) to f) = 1 mm.

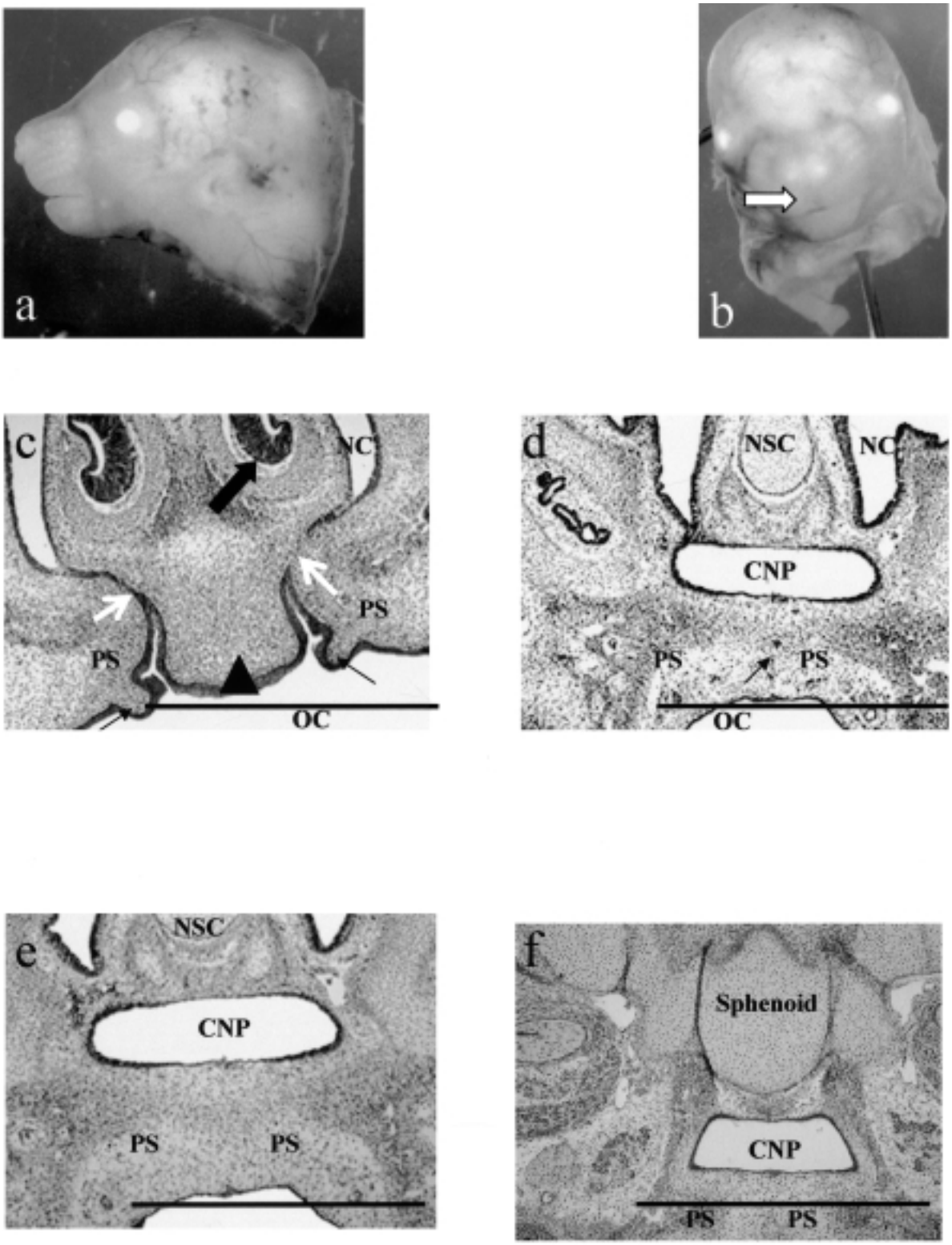

Figure 8 Group G 20mg kg^{-1} bw Acitretine, GD 8, 9, 10, 11

Fig. 8.- Group G (20 mg kg $^{-1}$ bw Acitretine, GD 8, 9, 10, 11). a) Macroscopic lateral view of the foetal head. x 4. b) Macroscopic frontal view of the foetal head. Arrow indicates cleft lip. x 4. c) Coronal section of an anterior region of the presumptive hard palate. Note that the nasal septum is between the palatal shelves (arrow-head). The palatal shelves (PS) have inferomedial outgrowths into the oral cavity (small arrows). On the right, there are the remains of an epithelial seam. On the left, fusion has commenced (white arrows). The black arrow indicates the Jacobson's organs. (OC = oral cavity; NC = nasal cavity). (H & E). d) Coronal section of the posterior section of the presumptive hard palate. The nasal septum is fused with the lateral nasal walls and the palatal shelves (PS) are fused with each other to form the CNP. Note remains of the Midline Epithelial Seam (arrow). (NSC = nasal septal cartilage; NC = nasal cavity; OC = oral cavity). (Masson's Trichrome). e) Coronal section of the posterior region of the presumptive hard palate. The palatal shelves (PS) are fused and the nasal septum (NSC) has fused with the lateral nasal walls forming the CNP. (Masson's Trichrome). f) Coronal section of the presumptive soft palate. Note the irregular formation of the sphenoid cartilage. The CNP is present. (PS = palatal shelves). (H & E). Scale bars for c) to e) = 1 mm.

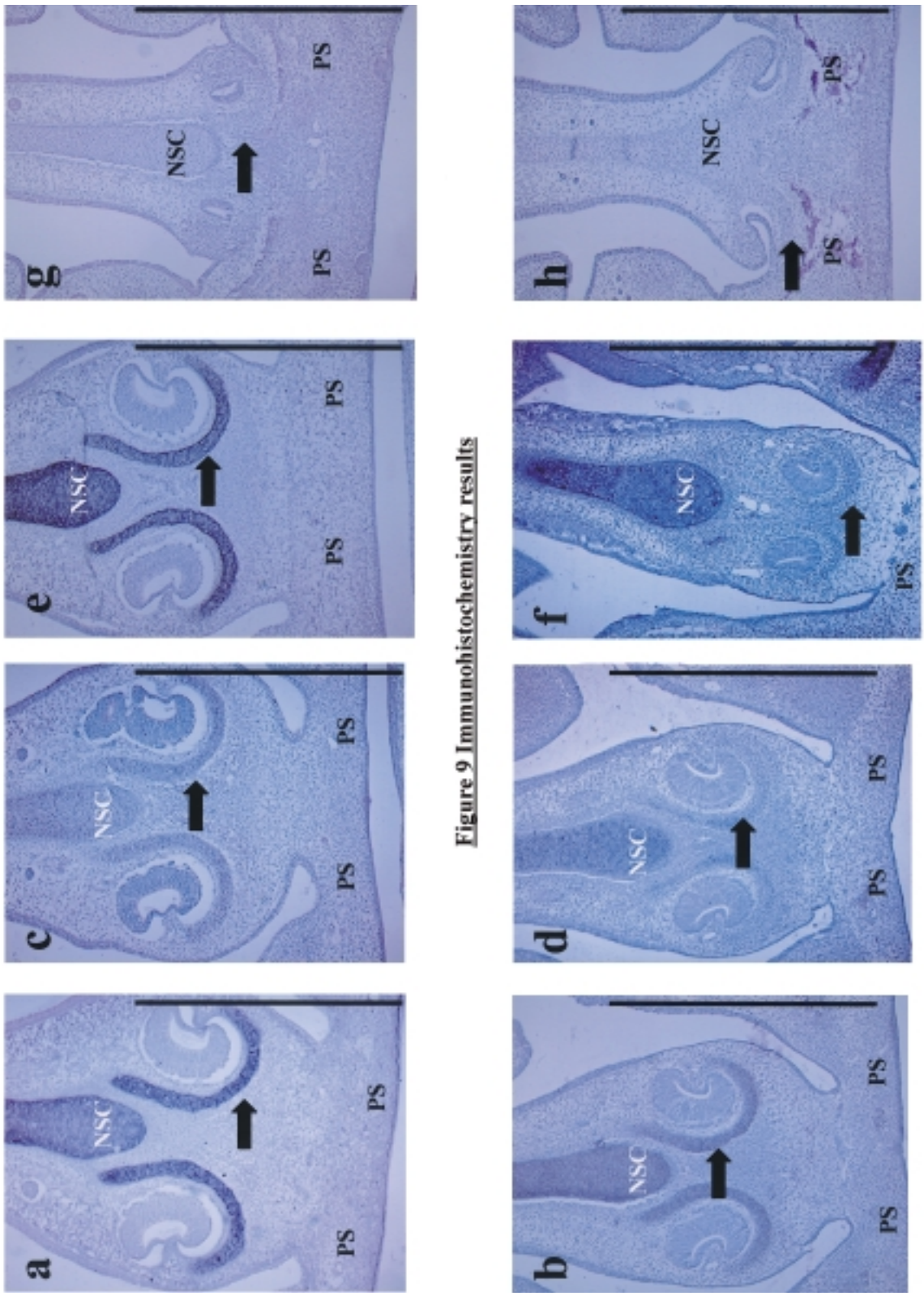

Fig. 9.- Immunohistochemistry Results. a) Control animal immunolabelled for chondroitin-4-sulphate using monoclonal antibody (MAb) 2B6 and counterstained with Haematoxylin. Note the intense label in the cartilage matrix of the nasal septum cartilage (NSC) and vomeronasal cartilage (arrow) and the lack of label in the matrix of the palatal shelves (PS). b) Group F sample immunolabelled for chondroitin-4-sulphate using MAb 2B6 and counterstained with Haematoxylin. Note the intense label in the cartilage matrix of the NSC and vomeronasal cartilage (arrow) and the lack of label in the matrix of the palatal shelves (PS). c) Control animal immunolabelled for G1 and chondroitin sulphate attachments using MAb 7D1 and counterstained with Haematoxylin. Note the intense label in the cartilage matrix of the NSC and vomeronasal cartilage (arrow) and the lack of label in the matrix of the palatal shelves (PS). d) Group F sample immunolabelled for G1 and chondroitin sulphate attachments using MAb 7D1 and counterstained with Haematoxylin. Note the label in the cartilage matrix of the NSC and vomeronasal cartilage (arrow) and the lack of label in the matrix of the palatal shelves (PS). e) Control animal immunolabelled for link proteins 1, 2 and 3 using MAb 8A4 and counterstained with Haematoxylin. Note the intense label in the cartilage matrix of the NSC and vomeronasal cartilage (arrow) and the lack of label in the matrix of the palatal shelves (PS). f) Group E immunolabelled for link proteins 1, 2 and 3 using MAb 8A4 and counterstained with Haematoxylin. Note the intense label in the cartilage matrix of the NSC and vomeronasal cartilage (arrow) and the lack of label in the matrix of the palatal shelves (PS). g) h) Negative control showing no autofluorescence and no non-specific binding. (Control and Group F) (NSC = nasal septal cartilage; PS = palatal shelves; arrow = vomeronasal cartilages). Scale bars = 1mm.

sition only in the specimens treated with all-trans RA. The effect of RA on these heads seems to have disrupted the ele-vation of the palatal shelves, although the mechanism(s) by which this occurred are presently unclear. RA treatment may have caused perturbations in the ECM, or disruption of the mesenchymal cells, preventing the organisation of the palatal shelf mesenchyme necessary for elevation (e.g. Moxham, 2003).

The present findings support previous studies that suggest that all-trans RA is the most teratogenic form of RA on the development of the rat palate (Emmanouil-Nikoloussi et al., 2000a, b), the most severe abnormalities being seen in animals treated with all-trans RA. On the other hand, the administration of 30 mg kg^{-1} bw of 13-cis RA appears to have had little adverse effect on the development of the palate, and acitretine (20 mg kg^{-1} bw) resulted in the fusion of the palatal shelves with the nasal septum and the separation of the oral and nasal cavities (the common nasal passage was, however, not oval in shape over the presumptive soft palate and the sphenoid cartilage was irregular in shape as compared with controls). These findings support the studies of Kraft et al. (1989) using 13-cis RA and Glineur et al. (1999) using etretinate (an analogue similar to acitretine).

Our results, focused on embryonic/foetal rat palatal development, support the view that the timing of administration of all-trans RA is more critical than its concentration, 30 mg kg^{-1} bw on GDs 8, 9, 10, 11 having more harmful effects on the development of the palate than 100mg kg^{-1} bw on GDs 8, 9. Treatment with all-trans RA also appears to elicit a more damaging effect on the development of the palate at GD 10-10.5 than with treatments at earlier stages. This contrasts with the suggestion of Ikemi et al. (2001), who suggested that the critical period for cleft palate formation by all-trans retinoic acid is on GD 14 in rats. Overall, our findings are consistent with those reported by Emmanouil-Nikoloussi et al. (2000a) but add significantly to the literature since the previous findings were recorded on GD 21 and here we observed teratogenic effects earlier on GD 18, when organogenesis has not been completed within the orofacial tissues.

Contrasting with the report of Emmanouil-Nikoloussi et al. (2000b), who reported ectopic cartilage in embryonic rat palate of excencephaly embryos, our present histological studies showed no evidence of cartilage formation within the palatal shelves of RA-treated rats on GD 18 without excencephaly. The morphology of the palatal tissues did not resemble that of the cartilage of the nasal septal cartilage, vomeronasal cartilage or Meckel's cartilage, which appeared to have formed normally in the RA-treated specimens. The nasal septal cartilage, vomeronasal cartilage and Meckel's cartilage were taken as positive controls for the immunohistochemical labelling of aggrecan within the specimens analysed, but the palate did not immunolabel for cartilage specific proteins. Our findings are thus not consistent with the hypothesis that RA causes ectopic cartilage formation within the developing rat palate. Future work to assess the inconsistencies between present and earlier reports could involve the additional use of antibodies to label other cartilage-specific ECM components (e.g. cartilage-associated collagens (type II)).

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