THE ROLE OF VITAMIN K IN CRANIOFACIAL DEVELOPMENT

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SUMMARY

The general aim of this thesis was to develop an animal model of the warfarin embryopathy. Exposure of pregnant rats to warfarin and vitamin K reproduced the hemorrhagic features associated with second and third trimester exposure. Similar treatment in the first 12 weeks of postnatal life reproduced the maxillonasal hypoplasia associated with first trimester exposure to warfarin. The abnormal facial development was associated with reduced growth and ectopic calcification of the nasal septal cartilage. These changes are thought to be related to the pharmacological effect of warfarin resulting in low fetal vitamin K levels and resultant abnormal vitamin K-dependent proteins.

Analysis of sectioned human embryos demonstrated that nasal septal growth is maximal during weeks 6 - 9 of gestation which is consistent with the observed critical period for the warfarin embryopathy.

A review of patients with maxillonasal hypoplasia indicated that other drug exposures may cause the phenotype of the warfarin embryopathy. Prenatal exposure to phenytoin was shown to be associated with a wide range in severity of this condition. Measurement of total prothrombin and PIVKA in the blood of newborns from mothers receiving anticonvulsant therapy showed that such infants had evidence of severe vitamin K deficiency particularly when exposed to carbamazepine.

It is proposed that any drug that causes the embryo/fetus to become vitamin K deficient during the 6-9 week critical period will cause the phenotype of the warfarin embryopathy. This phenotype should be called the vitamin K deficiency embryopathy to emphasise the common causal pathogenesis.

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I would like to thank Di Oakes and Trish Woodman-Brown for their assistance in the PIVKA and prothrombin assays reported in chapter 7. I would also like to thank The Boots Company and Roche Pharmaceuticals for their support in the supply of warfarin and vitamin K.

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Finally I would like to express my gratitude to my supervisor Bill Webster whose great love of science and sense of humour has made this thesis not only possible but also thoroughly enjoyable.

The conquest of the earth, which mostly means taking it away from those who have a different complexion or slightly flatter noses than ourselves, is not a pretty thing when you look into it too much.

(*The Heart of Darkness* Joseph Conrad).

DECLARATION

All the experiments described in this thesis were performed by myself in the department of Anatomy and histology, The University of Sydney. To the best of my knowledge this thesis does not contain material previously published or written by another person.

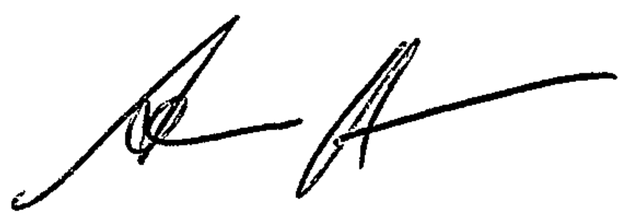


TABLE OF CONTENTS

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

LIST OF TABLES

CHAPTER 1

1.1 INTRODUCTION

This thesis addresses the issue of the role of vitamin K in mammalian development. The studies were initiated in an attempt to develop an animal model of the human warfarin embryopathy which was believed to be a consequence of warfarin-induced vitamin K deficiency in the first trimester of pregnancy. As the animal model was developed the studies were directed towards the role of vitamin K-dependent proteins in development and the identification of other drugs or conditions that might also cause prenatal vitamin K deficiency.

This introductory chapter includes a brief history of the discovery of vitamin K, its identification as an essential vitamin, and its relationship with warfarin. Research into the role of vitamin K has been inexorably linked to the discovery of warfarin, a potent and specific inhibitor of vitamin K recycling.

1.2 VITAMIN K

1.2.1 The discovery of vitamin K

Vitamin K is a one of the four fat soluble vitamins (A, C, E, K) and is contained in most leafy vegetables and may be produced as a precursor molecule by intestinal bacteria. It was identified as an essential vitamin by Henrik Dam in 1929. During experiments into the possible essential role of cholesterol in the diet he noted that chicks, fed a diet extracted by non-polar solvents, suffered subdural and muscular haemorrhages (Dam 1929). Furthermore, he noticed that blood taken from these animals clotted slowly. Studies in other laboratories demonstrated that the hemorrhages could not be prevented by administration of any of the known vitamins or other physiologically active lipids.

After further studies Dam proposed that there was a fat soluble antihemorrhagic vitamin which he called vitamin K from the German word koagulation, which was also the next letter of the alphabet not having previously been used to describe an existing or postulated vitamin

(Dam, 1935). In 1939 vitamin K was finally isolated from alfalfa (which had been shown to prevent hemorrhage in the chick) and characterised as 2-methyl-3-phytyl-1, 4-naphthoquinone (MacCorquodale et al., 1939). Since that time a number of compounds possessing vitamin K activity have been discovered and the term vitamin K is now used as a generic descriptor of 2-methyl-1, 4-naphthoquinone and all derivatives of this compound that exhibit antihemorrhagic activity in animals fed a vitamin K-deficient diet (Suttie, 1991). The compound 2-methyl-3-phytyl-1, 4-naphthoquinone is generally called vitamin K_1, or phylloquinone and is the common form of vitamin K found in animals and plants. The form of vitamin K extracted from putrefied fish meal is called vitamin K_2 and is one of a series of vitamin K compounds with unsaturated side chains called menaquinones which are formed by bacteria and are found in animal tissues. The parent compound of the vitamin K series is 2-methyl-1, 4-naphthoquinone and is called either vitamin K_3 or menadione.

1.2.2. The function of vitamin K

At the time of Dam's discovery only 2 blood clotting proteins had been defined, prothrombin and fibrinogen. Dam demonstrated that vitamin K-deficient chicks had reduced activity of prothrombin, and it was proposed that the haemorrhagic condition was due to a lack of plasma prothrombin (Dam et al., 1936). At this time the relationship between prothrombin and the conversion of circulating fibrinogen to a fibrin clot was poorly understood.

Early theories suggested that vitamin K was actually part of prothrombin but the true relationship between prothrombin and vitamin K was not established until the 1970s. It was demonstrated that vitamin K acts post-ribosomally in a metabolic step that converts a prothrombin precursor protein to active prothrombin. The precursor prothrombin (PIVKA II), was unable to bind calcium (Esmon et al., 1975a). The calcium binding peptides of prothrombin were shown to be glutamic acid residues that had been modified to γ-carboxyglutamic acid residues (Stenflo et al., 1974). Hence it was now established that the vitamin K-dependent step in prothrombin synthesis was the formation of γ-carboxyglutamic acid residues. It was shown that vitamin K was a cofactor for the microsomal

glutamyl carboxylase necessary for the carboxylation of glutamic acid (Esmon et al, 1975b).

It is now generally accepted that prothrombin is inactive or non-carboxylated when first formed and must be converted to the carboxylated form. This process is performed by a liver microsomal carboxylase that needs vitamin K as a cofactor. Prothrombin normally has a rapid turnover and in the absence of vitamin K normal or carboxylated prothrombin rapidly disappears and is replaced by the inactive or non-carboxylated form. Since this form of prothrombin cannot bind calcium ions it cannot take part in the blood clotting cascade and the affected animal becomes susceptible to hemorrhage. Prothrombin is known as a vitamin K-dependent protein.

It is now established that there are six other proteins involved in the blood coagulation mechanism that are also dependent on vitamin K for their synthesis. These are factors VII, IX and X involved in blood clotting and proteins C and S which have anti-coagulation activity. The sixth is protein Z and its function is unknown. All of these vitamin K-dependent proteins are continually produced in the liver and are used throughout the body for blood clotting (Suttie 1991).

At first it appeared that the only function of vitamin K was the carboxylation of proteins involved in blood coagulation. However, two additional vitamin K-dependent proteins that were not involved in blood clotting have been discovered. These are bone gla protein or osteocalcin (BGP) (Price et al., 1976) and matrix gla protein (MGP) (Price et al., 1983). Both of these proteins are carboxylated using vitamin K as a cofactor. These proteins are extra-hepatic in origin and are believed to play a role in the control of calcification in skeletal and other structures.

Hence at present the only known function of vitamin K is the post-translational carboxylation of glutamic acid (glutamyl) residues of certain proteins to provide sites for calcium binding.

1.2.3 Vitamin K nutritional requirements

Vitamin K levels in humans are reliant on dietary intake, especially the consumption of a few foods rich in the vitamin such as spinach, lettuce and broccoli. A daily intake range of 0.5 μg/kg to 1.5 μg/kg is sufficient to prevent any decrease in clotting factors and vitamin K deficiency does not normally occur in adult humans due to dietary deficiency (Suttie 1991).

The situation during pregnancy is more complex. It is well established that the developing fetus has much lower vitamin K levels than the mother (Mandelbrot et al., 1988). Hemorrhagic disease of the newborn has been a long-recognised syndrome (Lane et al., 1985) and is known to be vitamin K-responsive. In 1963 the American Academy of Pediatrics recommended prophylactic administration of vitamin K to all neonates in a attempt to prevent this condition. It remains unclear why the newborn has reduced vitamin K levels or whether this is in fact a deficiency; however the prophylactic administration of vitamin K to all newborn is still recommended.

1.3 WARFARIN

Much of the knowledge about vitamin K deficiency has come from clinical and experimental use of the potent vitamin K antagonist warfarin.

1.3.1 History of warfarin

Warfarin or more correctly sodium warfarin is one of a group of anticoagulant drugs known as coumarin derivatives. The discovery of the coumarin anticoagulants arose from observations that a hemorrhagic disease in cattle, which was prevalent in the American mid-west and western Canada in the 1920's, was linked to the consumption of improperly cured sweet clover hay (Campbell and Link, 1941). A contaminant of the hay was subsequently isolated and its chemical characteristics established (Campbell and Link, 1941; Stahmann et al., 1941). As an anticoagulant its significance as a treatment for thromboembolic disease was obvious, yet a yield of only 1g of coumarin per ton of clover meant that clinical usage was impractical. The contaminant was subsequently synthesised as a 3-substituted 4-

hydroxycoumarin which was chemically, physically, and biologically identical to the naturally occurring substance, and it could be prepared in abundant quantities and cheaply. It became available for clinical trials as an anticoagulant and was given the name dicoumarol (Meyer, 1959).

Subsequently another synthetic coumarin (3-(∂ phenyl-ß-acetylethyl)-4-hydroxycoumarin) was synthesised at the University of Wisconsin and was named warfarin for the Wisconsin Alumni Research Foundation. It was found that this anticoagulant was superior to dicoumarol as it could be given parentally, orally or rectally and the latency period was shorter than dicoumarol (Freeman and Myer, 1956). Warfarin remains the drug of choice to this day as it is inexpensive, effective, relatively safe and is the only anticoagulant that can be administered orally.

1.3.2 Mechanism of action of warfarin

As the investigation of the action of warfarin as an anticoagulant proceeded so to the action of vitamin K was established. It was found that vitamin K1 concentrate prepared from alfalfa could reverse the effect of dicoumarol in cattle (Link, 1945), and a few years later it was confirmed that vitamin K could counteract the action of dicoumarol in man provided that liver function was normal (Shapiro and Weiner 1949; Shapiro et al., 1943). It is now established that warfarin induces hemorrhage by creating an effective vitamin K-deficiency by blocking the hepatic microsomal enzyme vitamin K epoxide reductase which is necessary for intracellular vitamin K recycling (Suttie, 1991). The only known action of warfarin is to create a relative vitamin K-deficiency.

1.3.3 Therapeutic uses of warfarin

Warfarin has been used to treat or prevent many conditions where prolonged coagulation time is required to prevent unwanted blood clotting. These originally included deep venous thrombosis (primary prevention of deep venous thrombosis and established thrombosis), established pulmonary embolism, ventricular heart disease and atrial fibrillation, systemic embolism and transient ischaemic attacks, myocardial infarct, prosthetic heart valves and thromboembolic disease of pregnancy (O'Reilly, 1976).

Warfarin remains a commonly prescribed drug its only contraindication being pregnancy, even so many women remain on warfarin treatment during pregnancy when their physician considers its benefits outweigh its risks. There is no oral alternative to warfarin, heparin a parentally administered anticoagulant must be injected daily and is itself associated with fetal risk (Hall et al., 1980).

1.3.4 Risks of warfarin and pregnancy

By the 1960's advances in cardiovascular surgery (Starr et al., 1963) meant that patients with severe heart disease and those with congenital heart disease were for the first time reaching reproductive age. The introduction of prosthetic heart valves necessitated life-time warfarin maintenance to prevent thrombosis. If these patients became pregnant they needed to continue taking warfarin throughout pregnancy.

Von Sydow, in 1947 was the first to report hemorrhage as an adverse effect in the human fetus following the use of dicoumarol during pregnancy (Von Sydow, 1947). He reported a newborn with cerebral and generalised subcutaneous haemorrhage. By the mid-1960's hemorrhage , both fetal and maternal, was a recognised hazard of warfarin treatment during pregnancy. In a review of warfarin use in pregnancy in 1965 a perinatal mortality of 18.5% was reported (Villasanta 1965).

In 1966 there was a report of a child born to a mother receiving warfarin treatment following valvular surgery. The child who was blind and mentally retarded had nasal obstruction secondary to hypoplasia of the nasal structures. The author noted that although the fetal abnormalities described had not been previously associated with warfarin, he suggested they might be related (DiSaia, 1966). After a number of similar reports appeared in the medical literature the term warfarin embryopathy was proposed (Warkany, 1975).

1.4 WARFARIN EMBRYOPATHY

In a major review of anticoagulant use during pregnancy the criteria for warfarin embryopathy were considered to be exposure to coumarin derivatives in the first trimester and the presence of either stippled epiphyses or nasal hypoplasia. Other features such as central nervous system and eye anomalies were considered to be due to hemorrhage as a result of exposure in the 2nd and 3rd trimesters and were not part of the embryopathy (Hall et al., 1980). Since this review there have been many more case reports well tabulated and summarised by Schardein (1993).

In 1985 it was stated that warfarin is the only human teratogen that does not demonstrate its teratogenicity in a laboratory animal (Schardein, 1985). It was from this starting point that the studies described in this thesis were undertaken.

1.5 AIMS OF THIS THESIS

The initial aim of the studies described in this thesis was to establish an animal model for the human warfarin embryopathy. The results of the studies in which pregnant rats were exposed to warfarin are in chapter 2.

Since prenatal exposure to warfarin in the rat did not produce the facial changes typical of the warfarin embryopathy it was postulated that the critical period for facial development may take place postnatally in the rat. Hence rats were exposed postnatally to warfarin, these results are described in chapters three, four and five.

In the sixth chapter the normal development of the human nasal septum is examined and related to the animal model of warfarin embryopathy.

In the seventh chapter patients with maxillonasal hypoplasia are assessed and other causes of fetal vitamin K deficiency are suggested. Cord blood samples from control and anticonvulsant exposed newborn were assessed for vitamin K status. Carbamazepine was identified as a cause of vitamin K deficiency during pregnancy.

In summary a vitamin K deficiency embryopathy is discussed in chapter eight.

All animal experiments described in this thesis complied with the National Health and Medical Research Council of Australia guidelines on the care and use of animals for scientific purposes. The human data presented in chapter seven complied the National Health and Medical Research Council of Australia guidelines on human experimentation.

CHAPTER 2

2.1 INTRODUCTION

The anticoagulant coumarin derivatives, notably warfarin, are generally recognised as human teratogens (eg. Warkany, 1976; Hall et al., 1980; Schardein, 1985). It is interesting to note that identification of this teratogen was made by clinical observation (Di Saia, 1966; Kerber et al., 1968) since coumarin anticoagulants are the only well-established human teratogens that have not demonstrated teratogenicity in laboratory animals (Schardein, 1985).

In a major review of anticoagulant use during pregnancy (Hall et al., 1980) there were 418 reported pregnancies in which coumarin derivatives had been used. One sixth resulted in abnormal liveborn infants, one sixth in abortion or stillbirth and, at most, two-thirds in apparently normal infants. The authors subdivided the adverse effects of warfarin on the developing conceptus into three general groups. Exposure during the first trimester, particularly weeks 6 - 9, was associated with nasal hypoplasia, stippled epiphyses and hypoplasia of the extremities, a condition called the warfarin embryopathy. Exposure during the second and third trimesters was associated with anatomical abnormalities of the CNS presumably secondary to hemorrhage. These included microcephaly with cerebral agenesis, ventricular dilatation, Dandy-Walker malformation and midline cerebellar atrophy, and optic atrophy. Sequelae from these CNS anomalies included a very high incidence of mental retardation, blindness, spasticity, seizures and deafness. The third group, were those who had CNS problems due to late prenatal, perinatal or neonatal hemorrhage. Included in this group are those with intraventricular hemorrhage (Stevenson et al., 1980). Depending on the duration of the warfarin therapy, an affected infant may have features from all of these groups.

Warfarin is a small, water soluble molecule, that freely crosses the placenta (Quick 1946). In the human, it has been used during pregnancy to treat a variety of cardiovascular conditions by increasing the prothrombin time and decreasing the likelihood of clotting. It is this type of therapeutic use during pregnancy that is associated with the warfarin

embryopathy. However, despite the fact that warfarin has similar anticoagulant effects in cattle, rabbits, rats, mice, guinea pigs and dogs (Bingham et al., 1941) exposure in pregnant dogs (Quick, 1946), mice (Roll and Baer, 1967; McCallion et al., 1971; Kronick et al., 1974) rats (Beckman et al., 1982) or rabbits (Kraus et al., 1949; Knake and Vilmar 1963; Hirsh et al., 1970; Grote and Weinmann, 1973) does not induce fetal malformations although it may cause fetal death.

Our preliminary studies in the pregnant rat showed that the highest dose of warfarin tolerated by the dam (0.15 mg/kg/day) did not affect fetal development. Since warfarin inhibits vitamin K recycling the apparent difference in sensitivity between humans and experimental animals may be related to normal fetal and maternal vitamin K levels. Human fetuses have exceptionally low blood vitamin K concentrations, with a midgestation mean value of 30 pg/ml, and a maternal level of 395 pg/ml (Mandelbrot et al., 1988), compared with plasma levels of 8,600 pg/ml in 20-day rat fetuses and maternal levels of 22,000 pg/ml (Guillaumont et al., '88). However, a more recent publication has suggested that adult rat vitamin K levels are 2500 pg/ml (Yamanaka et al., 1990). If these measurements are accurate it may be much easier to produce vitamin K deficiency in the human fetus than in the rat fetus.

The failure of the highest tolerated warfarin dose in the pregnant rat to duplicate the human condition led us to investigate alternative methods. Subsequent investigations were based on a regimen introduced to study vitamin-K deficiency (Price and Williamson, 1981). They treated non-pregnant rats with massive daily doses of warfarin and counteracted the otherwise lethal effects of warfarin on blood coagulation by daily injections of vitamin K1. We used this regimen in pregnant rats to investigate whether there might be differential transfer of warfarin and vitamin K1 to the fetuses. When we treated pregnant rats in this way, the dams survived with a normal clotting time, but a number of the fetuses had severe hemorrhages when examined on day 21 of gestation.

2.2 MATERIALS AND METHODS

Sprague-Dawley rats obtained from Castle Hill Animal House, University of Sydney were mated overnight and examined the next morning by vaginal smear. Rats with a sperm positive smear were separated, and this day was considered day 0 of gestation. The rats were given Allied rat and mouse cubes and tap water ad libitum and were housed under a controlled 12/12 hour light/dark cycle.

2.2.1 Preliminary studies

Studies using male rats were performed to determine the highest daily oral dose of warfarin that could be tolerated. Male rats died within 7 days on a daily dose of 2 mg/kg but survived 21 daily doses of 1 mg/kg of warfarin. One non-pregnant female rat tolerated a similar dose for 21 days. Nine pregnant rats were treated with 1 mg/kg/day starting on day 9 of gestation but all died within 6 days. Four pregnant rats were given a single oral dose of 1 mg/kg on day 6 of gestation and 0.25 mg/kg/day subsequently but all died in the next 5 days. Three pregnant rats were given 0.15 mg/kg/day from day 8 until day 20. One rat died on day 16, but the other 2 survived to day 21 when they were killed and the fetuses examined. There was one litter of 16 and one of 15, there were no resorptions and all the fetuses appeared normal.

Subsequent studies were based on the regimen of administering both warfarin and vitamin K1. Preliminary studies showed that pregnant rats survived doses of warfarin up to 100 mg/kg/day throughout pregnancy providing they were given concomitant vitamin K1 (10 mg/kg/day). The regimen of combined administration of warfarin and vitamin K1 was then used as the basic treatment for a study of the teratogenic effects of warfarin.

2.2.2 Stage of pregnancy study

Pregnant rats were lightly anaesthetised with ether and given a daily intramuscular injection of vitamin K1 (10 mg/kg) (Kanakione - Roche Pharmaceuticals) and intubated with 100 mg/kg of sodium warfarin

(supplied and assayed by Boots Company, North Rocks, Sydney) dissolved in sterile water at 100 mg/ml. Two groups of rats were tested with dosing at different stages of pregnancy. Group 1 consisted of 5 rats dosed from day 1 to day 12 of gestation and group 2 consisted of 6 rats dosed from day 9 to day 20 of gestation. The pregnant rats were killed on day 21 of gestation and the fetuses examined for external malformations or signs of hemorrhage. The fetuses were then fixed in Bouins fluid and examined by razor sectioning for other signs of abnormality or hemorrhage. Four fetuses with overt cerebral hemorrhage and four control fetuses were fixed in alcohol and stained with alizarin red S and alcian blue (Kimmel and Trammel, 1981). Maternal blood clotting times were determined using an automated Quick's test on blood samples taken from the ether anaesthetised rat immediately before death on day 21

In an attempt to determine whether a higher dose of warfarin would be teratogenic when administered from days 1 - 12, 4 rats were given 200 mg/kg/day and 10 mg vitamin K1/kg/day. One of these rats was not pregnant, 2 died within 48 hours and one survived to day 21 with 10 normal fetuses and 6 resorptions. Two rats were given 500 mg/kg/day plus 10 mg/kg/day vitamin K1 both died within 48 hours.

2.2.3 Dose response study

Pregnant rats were treated from day 9 to day 20 of gestation with daily doses of both vitamin K and warfarin. The daily dose of vitamin K1 was kept constant at 10 mg/kg/day while the warfarin was tested at 50, 25, 12, 6, 3, and 1 mg/kg/day. Control animals consisted of a group of untreated pregnant rats and a group which were anaesthetised, given a daily injection of vitamin K1, and intubated with saline. All the rats were killed on day 21 of gestation. The number of resorptions and dead fetuses was recorded and each fetus was weighed and measured along its crown-rump length. The fetuses were examined for external malformations or hemorrhage, fixed in Bouins fluid and subsequently examined by razor sectioning for internal hemorrhage. When there was uncertainty about the presence of hemorrhage the razor sections were processed, embedded in paraffin, sectioned and stained with haematoxylin and eosin.

2.2.4 Statistics

For statistical analysis the litter was considered to be the experimental unit and the percentages of abnormal fetuses and resorptions between control and experimental animals was compared by Mann-Whitney U-test. Fetal weights, mean litter size and crown-rump lengths were compared using analysis of variance.

2.3 RESULTS

2.3.1 Stage of pregnancy study

Daily doses of 100 mg/kg of warfarin in conjunction with 10 mg/kg vitamin K1 had no apparent deleterious effect on the dams. Maternal prothrombin times were the same for control (20 and 20 secs) and experimental rats (17, 18 and 18 secs). There were no examples of hemorrhage in the dams and all treated rats maintained their pregnancies. The results presented in Table 2.1 show that warfarin and vitamin K1 exposure during the first 12 days of pregnancy caused some fetal growth retardation and one fetus out of 75 had an intraventricular hemorrhage. In contrast, exposure to 100 mg/kg warfarin and 10 mg/kg vitamin K from day 9 to day 20 of gestation caused a high incidence of hemorrhage in the fetuses and an increase in resorptions and a significant decrease in mean litter size. Thirty-six percent of the live fetuses had externally visible hemorrhages; many of which were subcutaneous. Other, more severe hemorrhages, seen at this dose of warfarin and at lower doses, affected the brain (Figs. 2.1 a and 2.1 c), the face (Figs. 2.1 b and 2.1 e), the eye (Figs. 2.1 g) and occasionally the limbs. Hemorrhages of the brain, mostly intraventricular, were seen in 7 out of 54 fetuses (13%). There were no external malformations other than changes associated with hemorrhage, such as hydrocephalus (Fig. 2.1 a) and there were no malformations or hemorrhages in the controls.

2.3.2 Dose response study

Following the stage of pregnancy results pregnant rats were only treated with warfarin and vitamin K1 on days 9 to 20. The dose of warfarin was progressively reduced in an attempt to find the no-effect level. The results are presented in Table 2.1. The only significant adverse effect of the treatment was hemorrhage in the fetuses, particularly intraventricular hemorrhage (Figs. 2.2 a, b and c). There were no significant differences in the incidence of externally visible or intraventricular hemorrhage between warfarin doses of 100, 50, 25, 12, 6 or 3 mg/kg. At 1 mg/kg the incidence of hemorrhage dropped dramatically with only 1 out of 40 fetuses affected. This result was not significantly different from controls, and was considered to be the no-effect level. Sections through the

internal ear of fetuses revealed hemorrhage affecting the spiral ganglia in 6 out of 50 fetuses examined from the seven warfarin treatments. There were no hemorrhages or external malformations in the controls.

Table 2.1 Effect of warfarin and vitamin K administration on pregnancy outcome in the Sprague-Dawley rat

Warfarin treatment (mg/kg)	Duration of treatment (gestational days)	No. of litters	Mean litter size	Percentage of implants resorbed	No. of dead fetuses	Percentage of live fetuses with ext. hemorrhage	Number of litters with hemorrhagic fetuses	Percentage of live fetuses with i.v.[1] hemorrhage	Mean CR length of live fetuses (mm)	Mean weight of live fetuses (g)
100	1–12	5	15.0 ± 3.4	0.0	1	0.0	0/5	1.3	37.0 ± 1.7	4.0 ± 0.3
100	9–20	6	9.0 ± 5.4*	14.8	1	36.3*	4/6	12.3	40.4 ± 1.9	4.5 ± 0.4
50	9–20	4	13.0 ± 4.1	0.0	0	33.8*	3/4	17.8**	38.5 ± 2.0	4.4 ± 0.3
25	9–20	5	14.8 ± 1.9	1.6	0	37.0***	5/5	22.8**	40.0 ± 1.2	4.7 ± 0.4
12	9–20	5	14.2 ± 2.7	0.0	1	24.2***	5/5	11.1*	39.4 ± 1.3	4.7 ± 0.6
6	9–20	5	13.2 ± 5.2	10.0	0	16.3***	5/5	6.5	40.3 ± 1.3	5.0 ± 0.4
3	9–20	3	15.0 ± 1.0	0.0	1	28.0**	3/3	13.8*	39.7 ± 0.3	4.9 ± 0.4
1	9–20	3	13.3 ± 0.6	0.0	0	2.7	1/3	2.6	38.2 ± 2.7	4.3 ± 0.6
0[2]	9–20	6	15.0 ± 2.4	0.0	0	0.0	0/6	0.0	40.0 ± 0.9	4.7 ± 0.3
Untreated		5	11.6 ± 5.6	0.0	0	0.0	0/5	0.0	40.0 ± 3.0	5.0 ± 0.7

[1]i.v.–intraventricular hemorrhage.
[2]Vitamin K and ether control.
*Significantly different from controls (0 mg/kg), $P<0.05$.
**$P<0.01$.
***$P<0.001$.

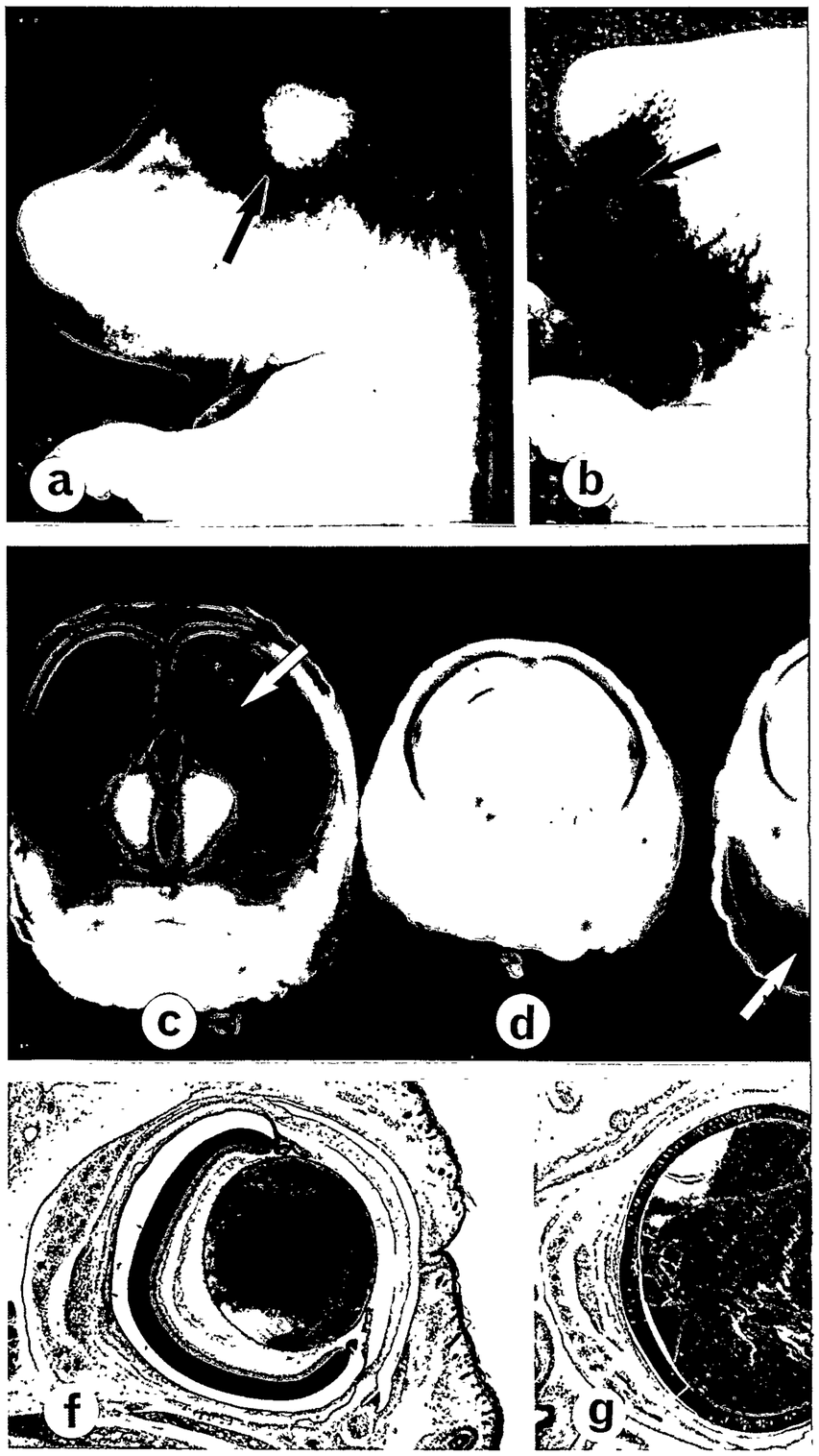

a
b
c
d
f
g

Figure 2.1

a Twenty-one day fetus with severe hydrocephalus due t
intraventricular hemorrhage (arrow). The dam recei
mg/kg/day and vitamin K1 10 mg/kg/day from day 9

b Twenty-one day fetus with a submandibular and latera
hematoma (arrow) from the same litter as the fetus in

c Razor section through the fetus shown in figure 2.1 a.
massive hemorrhage in the lateral ventricles (arrow) a
compression and destruction of the cerebral hemisphe

d Razor section through the head of a control fetus.

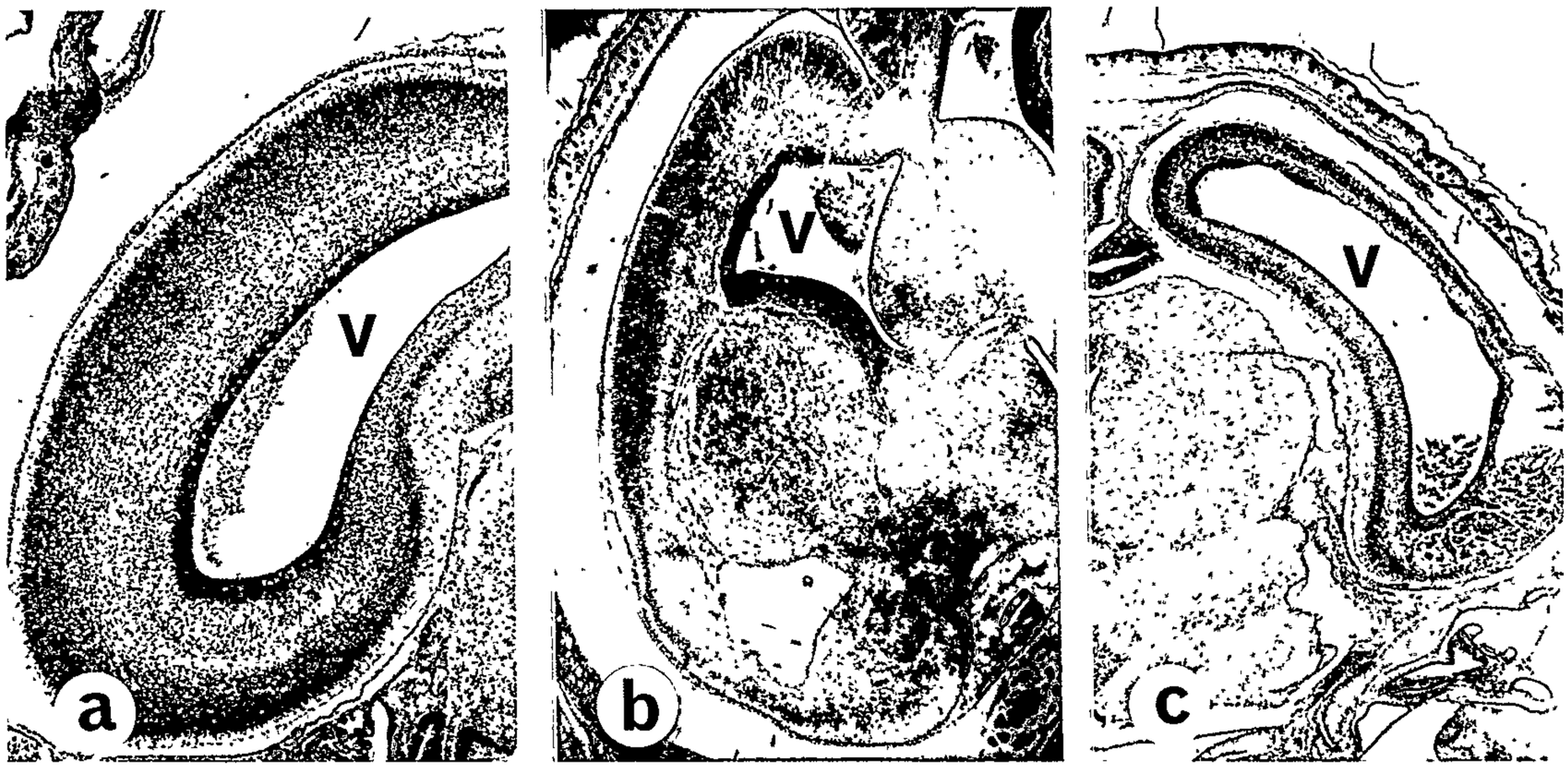
e Razor section through the head of the fetus shown in f
showing the bilateral submental and lateral facial hem
The brain is unaffected.

f Coronal section through the eye of a control 21-day fetu

g Coronal section through the eye of a 21-day fetus who
received warfarin (25 mg/kg/day) and vitamin K1 (10m
day 9 to day 20 of gestation. Note the hemorrhage in t
of the eye and compression and vacuolization of the le
compression and destruction of the retina.

Figures 2.2

a, b and c Paraffin sections through the heads of 21-day fetuses from dams who received warfarin and vitamin K1 from day 9 to day 20 of gestation. Note the hemorrhage in the lateral ventricles (v) and in figures b and c the destruction and cavitation of the brain tissue.


V
a
V
b
V
c

2.4 DISCUSSION

This study has shown that warfarin exposure in conjunction with vitamin K supplementation during prenatal development of the rat causes intraventricular hemorrhage in the fetuses, in some cases resulting in hydrocephalus. Localised hemorrhage in the walls of the cerebral hemispheres caused restricted areas of brain destruction, and hemorrhages in the eye and ear were similarly associated with tissue distortion and destruction. Intraventricular hemorrhage, microcephaly with cerebral agenesis, ventricular dilatation, Dandy-Walker malformation, optic atrophy, mental retardation, blindness, spasticity, seizures and deafness are all associated with prenatal exposure in the human.

Other, important features of prenatal warfarin exposure in the human, such as nasal or digital hypoplasia were not seen in the fetuses from the prenatally treated rats. A small number of day 21 fetuses with severe hemorrhage were stained with alizarin red but there was no evidence of bone stippling of other bony abnormalities. In the preliminary studies some prenatally treated rats were allowed to litter; the newborn pups appeared very bruised at birth and most died within 24 hours. Four pups from one litter were successfully raised and these were normal in appearance although it is possible that the snout reductions were not noted. At this stage we had expected the striking facial abnormality seen in the human newborn.

The most likely explanation for the absence of bony abnormalities following prenatal warfarin treatment in the rat is that growth and development of the bones is essentially a postnatal phenomenon in the rat with ossification of the nasal bones starting about 3 or 4 days before birth (Donaldson, 1924). In contrast, much of the growth of the facial bones in the human is prenatal with calcification of the nasal bones starting at about the 9th or 10 week of gestation (Norback and Robertson, 1951). Similarly, growth of the digits is almost exclusively a postnatal phenomenon in the rat while in the human extensive growth occurs prenatally.

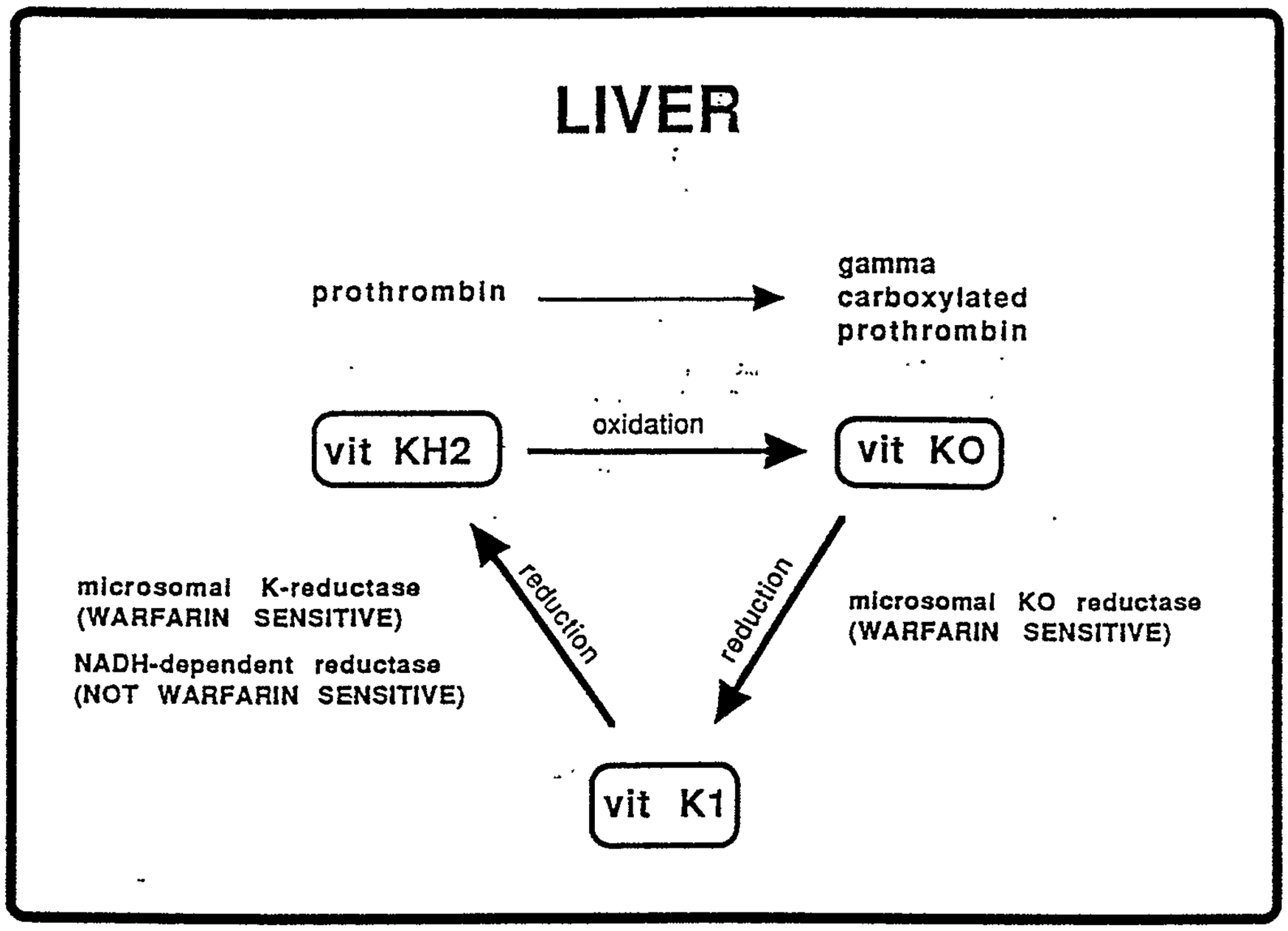
The teratogenic effect of warfarin is likely to be a consequence of the inhibition of vitamin K-dependent processes during development. This

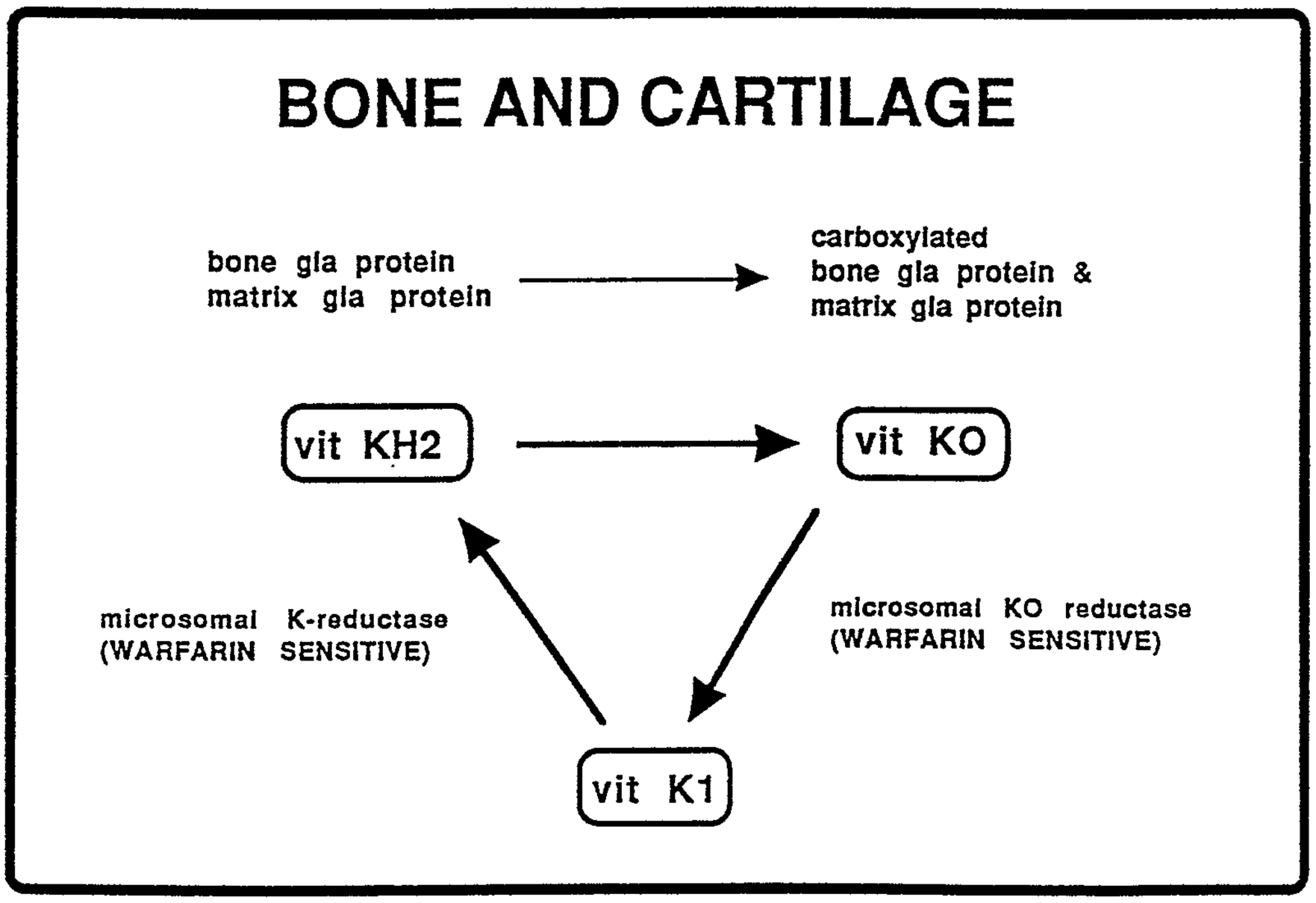
is supported by the identification of an infant with a genetic disorder of vitamin K metabolism who was phenotypically similar to infants with the warfarin embryopathy (Pauli et al., 1987). Warfarin's primary pharmacologic effect is inhibition of microsomal vitamin K epoxide reductase; this prevents vitamin K recycling and results in an effective vitamin K1 deficiency (Suttie, 1984). The specific action of vitamin K is as a cofactor in the posttranslational carboxylation of glutamic acid residues in certain proteins. This conversion of glutamic acid to gamma-carboxyglutamic acid (gla) creates effective calcium binding sites on these proteins (gla proteins). When these proteins are noncarboxylated they are functionally defective because they cannot bind calcium (Stenflo et al., 1974; Hauschka and Carr, 1982).

In warfarin-treated rats, the induced vitamin K deficiency prevents the normal carboxylation of vitamin K-dependent clotting factors in the liver and the animal is unable to effectively coagulate blood. If warfarin-treated animals are given large doses of supplementary vitamin K1 at the same time as warfarin, production of normal coagulation factors can be restored (Price and Kaneda, 1987). This is achieved by the animal using a second reductase present in the liver, which is NADH-dependent and insensitive to the action of warfarin. The NADH-dependent reductase can convert vitamin K naphthoquinone (K1) to vitamin K hydroquinone (KH2) which can then be used as a cofactor for carboxylation. However, unlike the warfarin-sensitive microsomal reductase, the NADH-dependent reductase cannot convert the vitamin K epoxide (K0), formed during the carboxylation, back to vitamin K1, so the vitamin K is not recycled and vitamin K epoxide accumulates. The warfarin treated rat can be kept alive for as long as vitamin K1 is administered. The second reductase is only present in the liver (Ulrich et al., 1988) so that vitamin K1 supplementation cannot antagonise the effect of warfarin on other non-hepatic vitamin K-dependent proteins, such as osteocalcin and matrix gla protein (MGP) (Price and Kaneda, 1987) or presumably any of the vitamin K-dependent proteins formed in the fetus (Fig. 2.3). Abnormal formation of osteocalcin and MGP in the fetus may be the underlying cause of the bony defects seen in the warfarin embryopathy but their precise role in bone and cartilage development remains obscure (Hauschka et al., 1989).

Figure 2.3

The difference in vitamin K recycling in the liver and bone and cartilage are illustrated. In the liver a NADH-dependant reductase allows carboxylation in the presence of warfarin provided a sufficient level of vitamin K1 is present. This warfarin insensitive reductase is absent in bone and cartilage and carboxylation cannot proceed in the presence of warfarin.


LIVER
prothrombin
gamma
carboxylated
prothrombin
vit KH2
oxidation
vit KO
reduction
reduction
microsomal K-reductase
(WARFARIN SENSITIVE)
NADH-dependent reductase
(NOT WARFARIN SENSITIVE)
microsomal KO reductase
(WARFARIN SENSITIVE)
vit K1


BONE AND CARTILAGE
bone gla protein
matrix gla protein
carboxylated
bone gla protein &
matrix gla protein
vit KH2
vit KO
microsomal K-reductase
(WARFARIN SENSITIVE)
microsomal KO reductase
(WARFARIN SENSITIVE)
vit K1

The present study has demonstrated that in the pregnant rat the co-administration of warfarin and vitamin K1 has no deleterious effect on the dams, which have normal blood clotting times, but causes hemorrhages in the fetuses which presumably have prolonged clotting times. Hemorrhage was associated only with treatment during the second half of gestation, corresponding in time with the development of capillary networks, particularly in the brain, and the development of a functional liver capable of producing clotting factors and a dependence on these factors. The incidence of fetuses with hemorrhages was not increased by larger doses of warfarin suggesting that doses of 3 mg/kg or above completely inhibit the fetal vitamin K epoxide reductase. The rat fetuses may be somewhat less sensitive to warfarin than gravid rats as 1 mg/kg/day was lethal within 5 days to pregnant rats unprotected by supplementary vitamin K.

It was initially thought that the warfarin embryopathy was caused by hemorrhages in the CNS and microhemorrhages in the nasal cartilage and epiphyses due to the effect of warfarin on prothrombin (Shaul et al., 1975; Shaul and Hall, 1977). Against this theory was the observation that in the human the critical period of exposure to warfarin was 6-9 weeks of gestation which was before coumarin sensitive clotting factors were present in the embryo. In the human vitamin K-dependent clotting factors do not develop until 12 to 14 weeks gestation (Bleyer et al., 1971).

There seem to be three main possibilities to explain why there is hemorrhage in the rat fetuses but not in the dam. Firstly, it is possible that both warfarin and the excess vitamin K1 enter the fetus but the fetus does not have a NADH-dependent reductase in its hepatocytes so it cannot utilise the extra vitamin K1 to overcome the warfarin inhibition of vitamin K epoxide reductase. However, hemorrhage has not been a feature of neonatally treated rats suggesting that a NADH-dependent reductase is present immediately after birth. Wallin has demonstrated that in the rat the NADH dependant non-warfarin-sensitive reductase emerges in the E17 fetal liver and at birth its levels are low, shortly after birth its activity dramatically increases to attain adult levels on day 5 (Wallin 1989). The second option is that there is differential transfer of warfarin and vitamin K1 to the fetus so that there is insufficient vitamin K1 in the fetus to counteract the effect of the warfarin. The NADH-

dependant reductase needs high levels of stored vitamin K1 to function. There have been studies in both the human (Mandelbrot et al., 1988) and the rat (Hamulyak et al., 1987; Guillaumont et al., 1988) which have demonstrated that although vitamin K1 crosses the placenta a very large maternal-fetal gradient exists for endogenous as well as pharmacological levels. The third possibility is that the warfarin present in the dam prevents adequate transfer of vitamin K1 to the fetuses so that the fetuses are vitamin K deficient. In each case the carboxylation of vitamin-K dependent clotting factors in the fetus would be deficient. At present we do not know whether some or all of these mechanisms are involved.

The apparent absence of bony or other abnormalities in the day 21 fetuses suggests that the other vitamin K-dependent proteins are not necessary for normal prenatal growth in the rat, perhaps because most ossification takes place postnatally in the rat. However, although there were no gross growth or bony defects, smaller changes may have been undetected. Further studies will indicate whether postnatal exposure to warfarin in the rat will duplicate all features of the warfarin embryopathy and these results are presented in chapter 3.

CHAPTER 3

3.1 INTRODUCTION

In chapter 2 the effect of concurrent exposure to vitamin K and warfarin during prenatal rat development was examined. This regimen caused hemorrhages in the late term fetuses producing pathology that was consistent with that seen in human fetuses exposed to warfarin in the second and third trimesters. It did not cause nasal hypoplasia or bone stippling, which are seen after warfarin exposure during the first trimester in the human.

A likely explanation for this difference is that the critical periods for nasal and skeletal development occur prenatally , during the first trimester, in the human, while similar processes occur during late fetal or early postnatal life in the rat. For instance, in the rat, ossification of the skull, vertebrae and long bones all start on prenatal day 17 (Strong, 1925) just 5 days before birth. In the human, similar processes start during the sixth and seventh weeks of gestation.

To test this hypothesis, newborn rats were administered warfarin in conjunction with vitamin K1 for up to 12 weeks. This regimen permits the treated pups to produce normal prothrombin while creating an extrahepatic vitamin K deficiency (Price and Williamson, 1981). The treated pups developed a marked maxillonasal hypoplasia and other skeletal anomalies similar to those features seen in the human when exposed in the first trimester.

3.2 MATERIALS AND METHODS

Sprague-Dawley rats obtained from Castle Hill Animal House, University of Sydney were mated overnight and examined the next morning by vaginal smear. Rats with a sperm-positive smear were separated and allowed to litter. The day of birth was considered postnatal day 0. The rats were given rat and mouse cubes (Y.S.F. Pty Ltd, N.S.W.) and tap water ad libitum and maintained under controlled conditions.

3.2.1 Postnatal rat treatment

On postnatal day 1 each litter containing 10 or more pups was assigned to one of three experimental groups. For each litter in each treatment group, the litter size was reduced to 10 on postnatal day 14. When possible it consisted of 5 males and 5 females. Litters were weaned on postnatal day 21. Pups were killed at various times throughout the 12 weeks of the experiment for histological and biochemical analysis.

Group 1 consisted of six litters in which the pups were given a daily subcutaneous injection of 100 mg/kg sodium warfarin (supplied and assayed by Boots Company, North Rocks, Sydney) dissolved in distilled water at 100 mg/ml. At the same time the pups were given a daily subcutaneous injection of 10 mg/kg vitamin K1 (Konakione, Roche Pharmaceuticals). This regimen was continued for periods of up to 12 weeks. A total of 23 rats (11 males and 12 females) were kept until 12 weeks.

Group 2 consisted of three litters in which the pups were given a daily subcutaneous injection of 10 mg/kg vitamin K1 only. Twenty-one animals were kept for 12 weeks (11 males and 10 females).

Group 3 consisted of three untreated litters. Twenty-four animals were kept for 12 weeks (12 males 12 females).

3.2.2 Measurements

Each week the rat pups were measured (nasal length, tail length, total length, and weight). At 12 weeks the surviving rats were killed by carbon

dioxide inhalation. The head and right forelimb were stained with alizarin red and alcian blue (Kimmel and Trammel, 1981). The stained skull was measured (±0.5 mm) with a micrometer to determine the following dimensions (1) maximum skull length from anterior end of the nasal bones to the posterior border of the occipital bone, (2) nasal bone length, (3) frontal bone length, (4) parietal bone length, (5) interparietal bone length, (6) premaxilla length, (7) maxilla length, (8) mandibular length, (9) bizygomatic width, (10) maximum width of snout, (11) minimum transfrontal width, (12) facial height from first molar tooth to superior margin of frontal bone and (13) maximum nasal height. The maximum length of the scapula, humerus, ulna were measured (±0.5mm) using a micrometer, and the third metatarsal and third phalanx were measured (±0.1 mm) using an ocular micrometer. The measurements for each group were compared by analysis of variance using Dunnett's q value for a two-tailed test (Zar, 1984). After each skull had been measured the nasal septum was removed and examined for abnormalities, in particular signs of calcification.

3.2.3 Histology

Both experimental and control rats were killed at regular intervals after the start of treatment for histological examination. Non-decalcified nasal septa, tail vertebrae, proximal and distal femur segments were fixed in 10% formal saline and then embedded in Historesin (H & D Scientific Supplies, Sydney) and sectioned at between 1 and 3 micra. The sections were stained for calcium using von Kossa's silver technique (Drury and Wallington, 1976) and counterstained with 0.25% azure II in 0.5% sodium borate. Some sections were only stained with azure II.

3.4 RESULTS

3.4.1 Skeletal Measurements

The warfarin-vitamin K-treated rats were healthy and there was no evidence of hemorrhage. The external growth parameters (±SD) for the three groups at 12 weeks are presented in Tables 3.1 and 3.2. For both male and female warfarin treated rats there was a significant reduction in tail length (12-17%) and nasal length (7-13%) compared with the untreated or vitamin K-treated rats. There were also significant reductions in overall length (6-12%) and weight (7-13%).

The warfarin-treated rats had a characteristic facial appearance that distinguished them from controls. Their snout appeared shorter and broader and the pinna of the ear was reduced in size. These features were particularly evident after 3 weeks treatment (Fig. 3.2 a and b). Although the facial appearance was still characteristic after 12 weeks treatment (Fig. 3.2 c and f) it was less obvious than at 3 weeks.

Measurements of the alizarin-stained skulls after 12-weeks treatment (Table 3.3) showed that the warfarin-vitamin K treatment had a differential effect on growth of the various bones of the skull. Compared with controls, the skull length in the warfarin-treated rats was reduced by 5-6% in males and 3-6% in females. The anterior part of the skull was far more affected than the posterior part. Nasal bone length was significantly reduced by 12-13% in males and 14% in females while frontal bone length was reduced by 2-4% in males and 3-5% in females. For the parietal and interparietal bones differences between the warfarin and control groups were not significant. The premaxilla, which articulates along the entire length of the nasal bone, was also significantly reduced in length (5-7%) in the warfarin treated animals while the laterally related maxilla was reduced by 6-12%. Mandibular length was not significantly different in the males and was slightly larger in the warfarin-treated females.

The width of the snout (mostly width of the nasal bones) was significantly reduced in females by 6-8% and in males by 9-12%. There was also a small reduction in the minimum transfrontal width in the warfarin-treated rats. Facial height was significantly reduced in the warfarin group by 3-5%.

Measurements of the bones of the right forelimb (Tables 3.4 and 3.5) showed that the bones from the male and female warfarin-treated rats were slightly shorter than those of the controls, overall length was reduced by 4-5% in males and 4-5% in females. There was no evidence that the distal bones were more affected than proximal bones.

3.4.2 Nasal septum

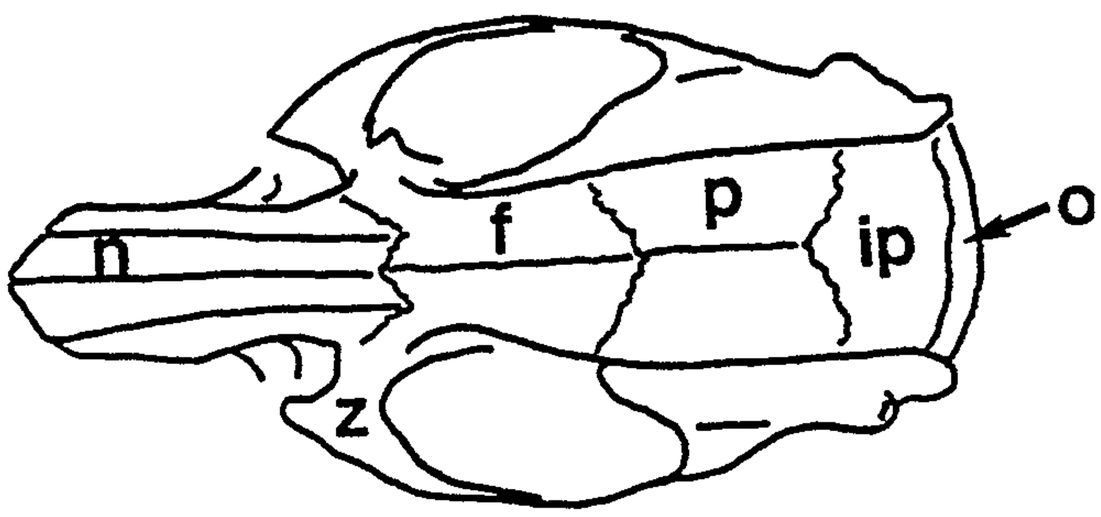
After skull measurements had been taken the skulls were disarticulated so that the nasal septum could be examined. The alizarin-stained nasal septa from control rats (both vitamin K-treated and untreated) showed no evidence of calcification in the septal cartilage. In contrast, all the septal cartilage from the 12-week warfarin-treated rats showed extensive areas of calcification (Fig. 3.3a). The abnormal sites of calcification were similarly localised in all the septa examined. There was a large area in the inferior half of the septal cartilage continuous with the adjacent palate and a narrow strip where the septum is in contact with the perpendicular plate of the ethmoid and nasal bones. The full height of the anterior septum was calcified but the calcification did not extend into the external nose. The calcium deposits appeared to be in the matrix surrounding the cells (Fig 3.3b) and this was confirmed by transverse sections through the septum stained by von Kossa's technique for calcium (Fig. 3.3 c and d). The calcification first appeared 3 weeks after the start of warfarin treatment as a small discrete area on the superior border of the septum and progressively increased in area during the following weeks of treatment. Additional experiments showed that the calcium deposits did not disappear after the cessation of treatment.

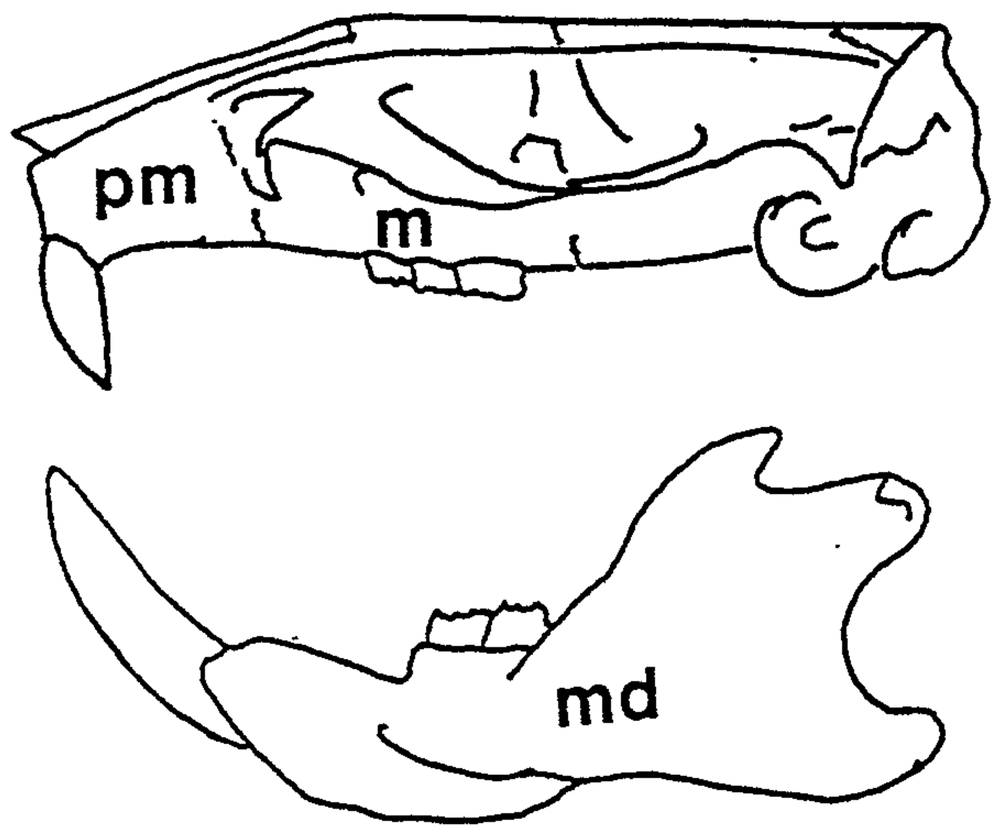
The alizarin-stained skeletons of warfarin-treated rats were examined at various times after the initiation of treatment in an attempt to determine whether there was any abnormal calcification in the limbs or axial skeleton that might correspond to the "stippling" described in the human warfarin embryopathy. Despite extensive examination, particularly in the first 14 days of treatment, there were no signs of stippling except the previously described calcification in the septal cartilage.

3.4.3 Histology

Sections of long bones and vertebrae were examined by light microscopy at various times after the initiation of warfarin treatment. The sections were either stained for calcium by von Kossa's technique or with azure II for normal histology. When the growth plates were examined in the femur or in the tail vertebrae from 12-week warfarin-treated rats many calcium "bridges" were seen to traverse the growth plate (Fig. 3.4 c and d), similar

bridges were not seen in controls (Figs 3.4 a and b). The bridges consist of ectopic calcification occupying the matrix of some longitudinal septa, these first appeared about postnatal day 10 when growth plates were established in the long bones and vertebrae. The bridging was seen in all warfarin-treated rats after that time. In addition to calcium bridges, disorganisation and reduction in the number of cells in the column of growth plates were sometimes observed. The primary and secondary ossification centres appeared to be normal.





Abbreviations

n	nasal
f	frontal
p	parietal
ip	interparietal
o	occipital
z	zygomatic
pm	premaxilla
m	maxilla
md	mandible

Table 3.1 Growth measurements in male rats (12 weeks)

Treatment	number of litters	number of rats	weight (g)	body length (mm)	tail length (mm)	nasal length (mm)
warfarin	6	11	311.1±20.2[1,2]	380.9±11.8[2,3]	168.5±8.1[2,3]	21.4±1.2[2,3]
vitamin K	3	11	334.6±22.8	406.8±9.0[2]	190.9±8.6[2]	23.8±0.6
untreated	4	13	344.8±34.2	424.0±18.3	201.8±9.6	24..5±0.8

[1] Significantly different from vitamin K group P<0.05

[2] Significantly different from untreated group P<0.01

[3] Significantly different from vitamin K group P<0.01

Significantly different from untreated group P<0.05

Table 3.2 Growth measurements in female rats (12 weeks)

Treatment	number of litters	number of rats	weight (g)	body length (mm)	tail length (mm)	nasal length (mm)
warfarin	6	12	207.3±18.1[1]	338.8±14.8[1,2]	152.8±9.4[1,2]	20.8±1.0[1,2]
vitamin K	3	10	237.4±37.2[3]	380.5±9.0	184.5±7.6	22.7±0.5
untreated	4	14	224.2±23.0	384.3±13.9	186.0±8.2	22.4±0.6

[1] Significantly different from vitamin K group P<0.01

[2] Significantly different from untreated group P<0.01

[3] Significantly different from untreated group P<0.05

Significantly different from vitamin K group P<0.05

Table 3.3. Skull measurements (mm) in 12-week rats

	males			females		
	warfarin	vitamin K	untreated	warfarin	vitamin K	untreated
number of rats	11	11	13	12	10	14
skull length	42.7±1.0[1,2]	45.2±0.9	45.4±1.8	41.3±1.2[1,2]	43.6±1.4	42.3±1.6
nasal bone length	15.2±1.1[1,2]	17.5±0.6	17.3±0.6	14.6±0.7[1,2]	17.0±0.7	17.0±0.6
frontal bone length	13.2±0.7[3]	13.8±0.6	13.5±0.6	12.4±0.5[1,4]	13.1±0.4	12.8±0.5
parietal bone length	7.8±0.8	7.6±0.6	7.7±0.7	7.5±0.3	7.1±0.7	7.4±0.4
interparietal bone length	6.3±0.6[4]	6.7±0.4	6.8±0.5	6.3±0.3	6.4±0.6	6.6±0.6
premaxilla length	10.7±0.5	11.5±0.4	11.5±1.6	10.4±0.6[3]	11.1±0.5	10.9±1.0
maxilla length	15.9±1.2[1,4]	17.7±0.7	17.2±1.7	14.9±1.5[1,4]	17.0±0.7[4]	15.9±0.7
mandibular length	24.0±0.9	24.5±1.1	24.8±1.3	24.1±1.0[4]	23.5±0.9	23.7±1.4
bizygomatic width	23.1±0.8	23.3±0.5	23.5±0.7	22.0±0.8	22.6±0.8	22.3±0.6
width of snout (max.)	8.5±1.9[2,3]	9.3±0.8	9.7±0.4	8.4±0.4[2,3]	8.9±0.4	9.1±0.6
transfrontal width (min.)	6.5±0.2[1,2]	6.9±0.3	6.9±0.3	6.4±0.3[4]	6.6±0.3	6.7±0.3
facial height	13.4±0.4[1,2]	14.0±0.5	14.1±0.3	12.8±0.3[1,4]	13.4±0.3	13.1±0.4
max. nasal height	8.1±0.4[4]	8.5±0.5	8.7±0.7	7.9±0.3[3]	8.3±0.4	8.0±0.5

[1] - significantly different from vitamin K group or untreated group respectively $P < 0.001$.

[2] - significantly different from vitamin K group or untreated group respectively $P < 0.01$.

[3] - significantly different from vitamin K group or untreated group respectively $P < 0.05$.

[4] – significantly different from untreated group $P < 0.05$.

Table 3.4 Forelimb bone length (mm) in 12-week male rats

treatment	number of rats	scapula	humerus	ulna	meta-carpal	proximal phalanx	middle phalanx	distal phalanx
warfarin	11	25.3±1.0[1]	25.8±0.6[1,2]	30.2±0.6[34]	8.2±0.7[3,4]	5.2.±0.2[1,3]	2.8±0.1[3]	2.7±0.1
vitamin K	11	28.8±12	27.0±0.5	31.6±0.6	8.5±0.2	5.6±0.2	3.0±0.1[4]	2.7±0.2
untreated	13	26.8±1.5	26.9±1.5	31.3±0.9	8.5±0.3	5.5±0.3	2.9±0.2	2.7±0.1

[1] - significantly different from vitamin K group or untreated group respectively P< 0.001.

[2] - significantly different from vitamin K group or untreated group respectively P< 0.01.

[3] - significantly different from vitamin K group or untreated group respectively P< 0.05.

[4] – significantly different from untreated group P < 0.05.

Table 3.5 Forelimb bone length (mm) in 12-week female rats

treatment	number of rats	scapula	humerus	ulna	meta-carpal	proximal phalanx	middle phalanx	distal phalanx
warfarin	12	23.4±1.2	24.0±0.8[1,2]	28.2±1.7[1, 2]	7.9±0.2[1,3]	5.2±0.3	2.6±0.1[1,2]	2.5±0.1[4]
vitamin K	10	24.4±0.9	25.2±0.4[2]	30.6±0.4	8.2±0.2	5.3±0.3	2.8±0.1	2.6±0.1
untreated	14	24.3±1.3	25.0±0.7	30.5±0.8	8.1±0.3	5.2±0.2	2.8±0.1	2.5±0.2

[1] - significantly different from vitamin K group or untreated group respectively P< 0.001.

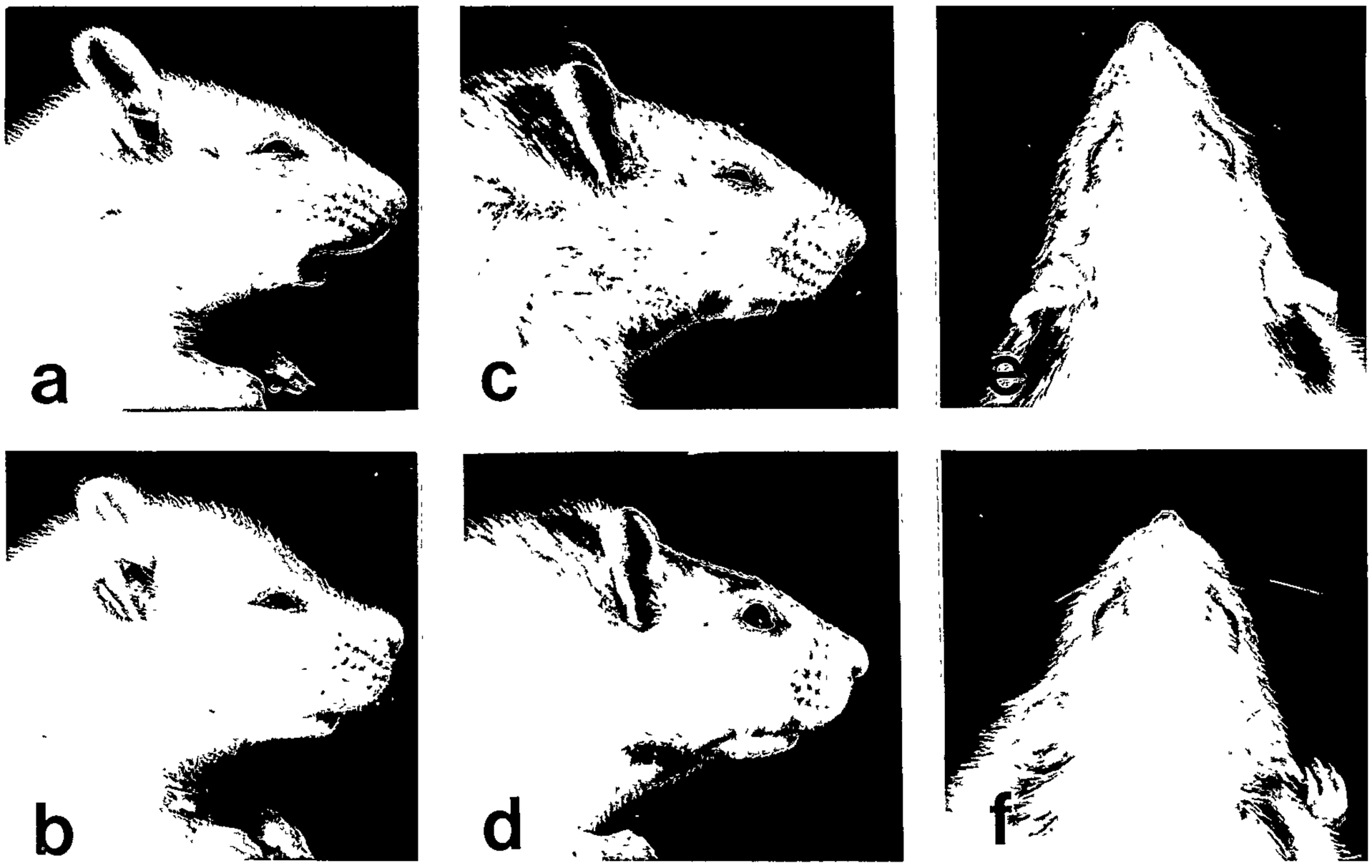
[2] - significantly different from vitamin K group or untreated group respectively P< 0.01.

[3] - significantly different from vitamin K group or untreated group respectively P< 0.05.

[4] – significantly different from untreated group P < 0.05.

Figures 3.2

a-f. Lateral view of a 3-week control (a) and warfarin-treated rat (b). The warfarin-treated rat has a distinctive facial profile characterised by a short, slightly "upturned" snout. Lateral view of a 12-week control (c) and warfarin-treated rat (d) and dorsal views (e and f) of the same rats. The warfarin-treated rat has a short slightly broader snout compared to the control.


a
c
e
b
d
f

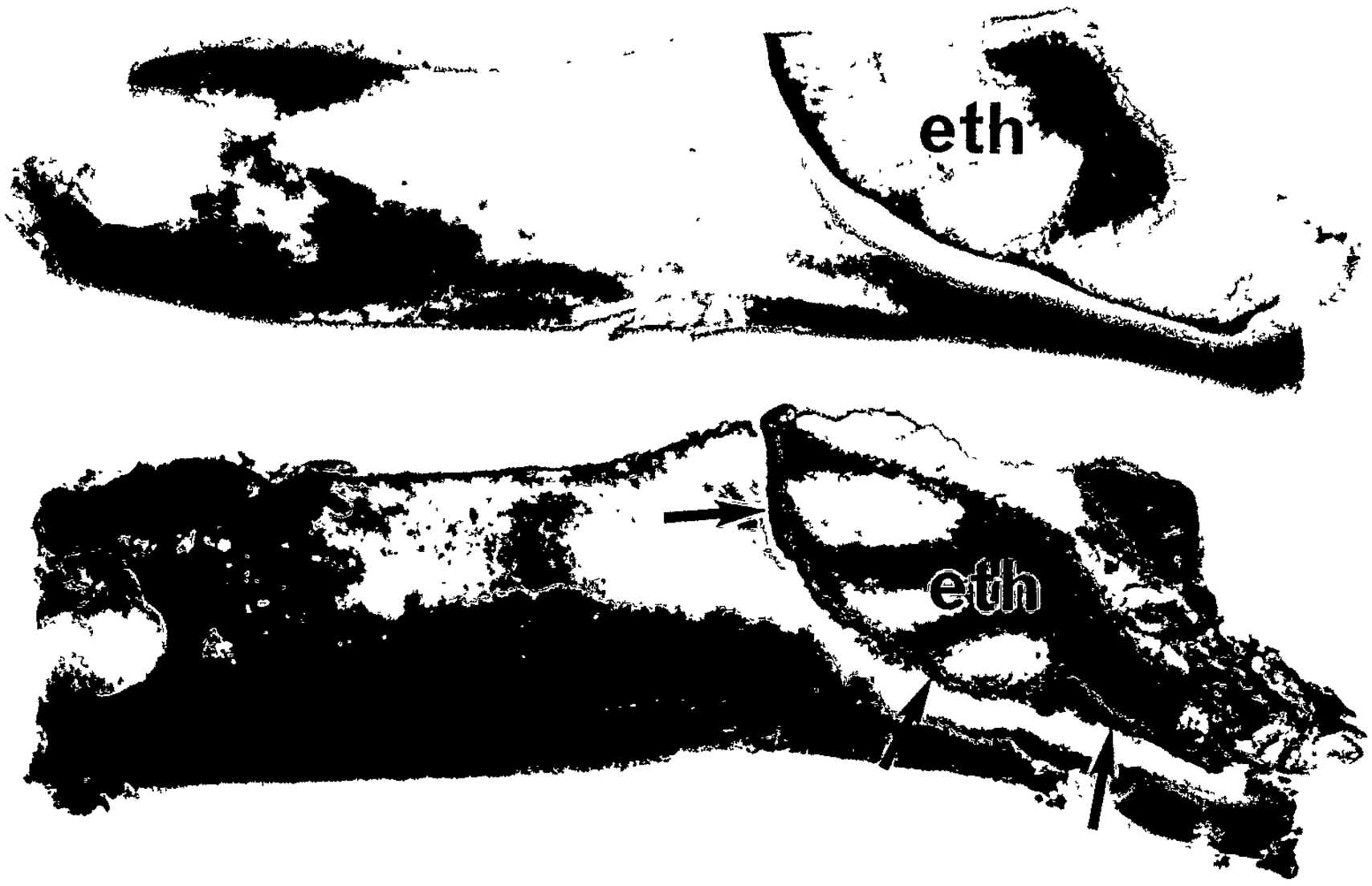
Figures 3.3

a Lateral view of the septal cartilage and perpendicular plate of the ethmoid stained with alizarin red and alcian blue from a 12-week control rat (upper) and a 12-week warfarin-treated rat (lower). There is no evidence of calcification in the septal cartilage from the control but in the warfarin-treated cartilage there are extensive alizarin stained areas (appear black in the photograph), particularly anteriorly and inferiorly. The cartilage is firmly attached to the perpendicular plate of the ethmoid by the abnormal calcification.

b An enlargement of the abnormal calcification seen in figure 3.3a. The calcium deposits form a distinct crystalline pattern around the chondrocytes. (x56)

c Transverse section through a nasal septum from a 12-week warfarin-treated rat. The section is stained with azure II and shows a distinct and difficult to cut (shatter lines) extracellular matrix between the chondrocytes. (x45)

d Transverse section through a nasal septum from a 12-week warfarin-treated rat. The section is stained with von Kossa's silver technique to demonstrate calcium. The darkly-stained, dense deposits seen intercellularly in the centre of the septum are presumably calcium. (x180)



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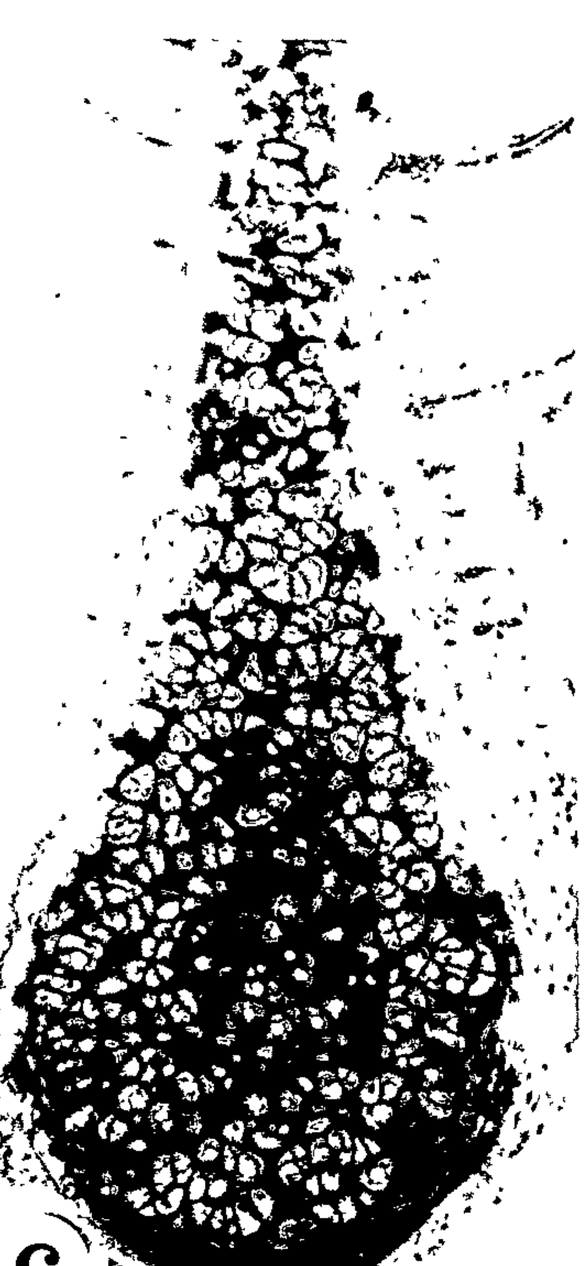

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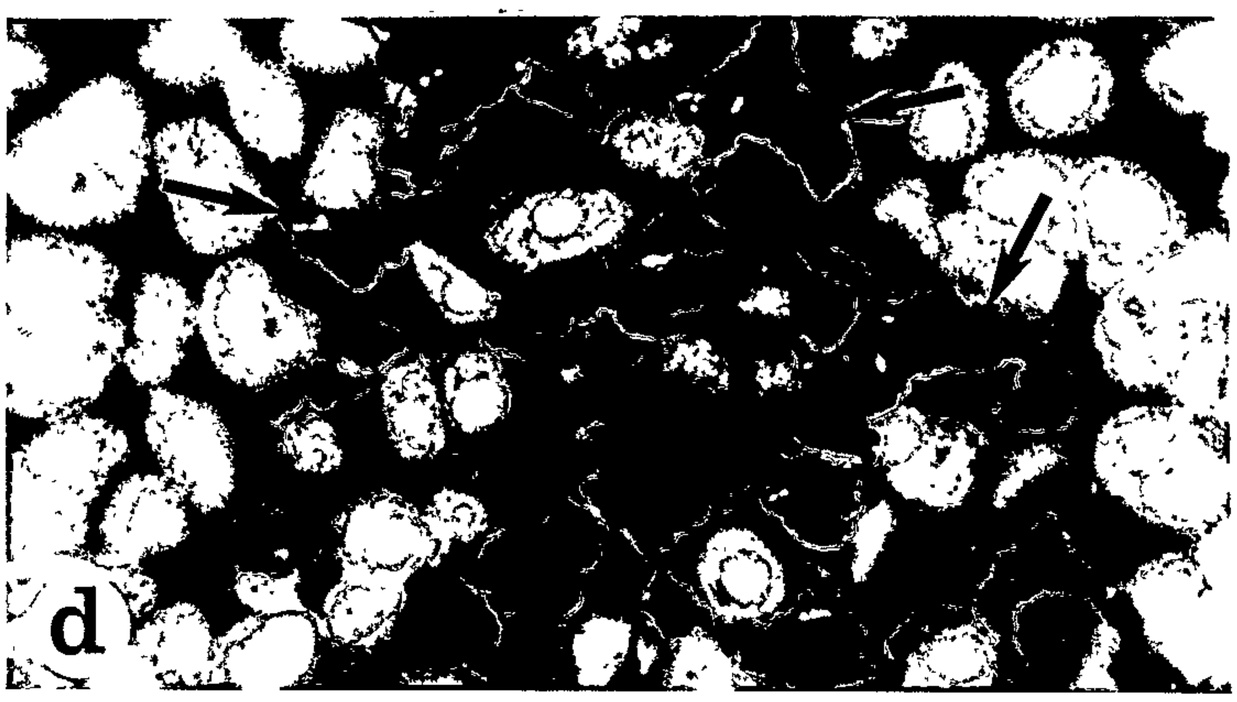


d

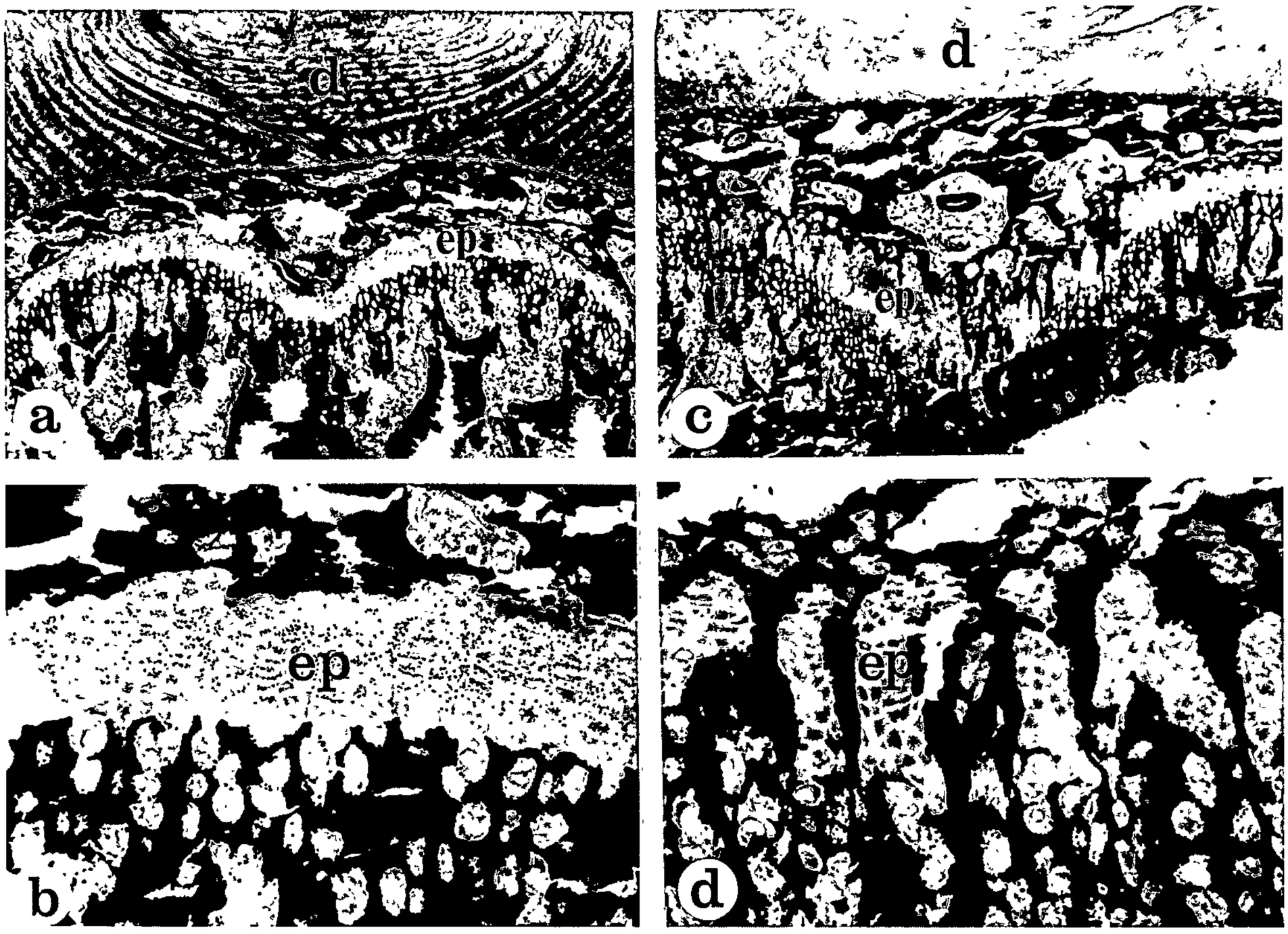
Figures 3.4

a Longitudinal section through tail vertebrae from a control 6-week rat stained by von Kossa's silver technique to demonstrate calcium. The epiphyseal growth plate (ep) is clearly visible and is unstained by the von Kossa's technique. Above the growth plate is the epiphyses (dark stained) and below the diaphysis, both have heavy stained heavily for calcium. d, vertebral disk. (x60)

b Enlargement of a similar section as seen in a., showing the epiphyseal growth plate (ep). (x300)

c Longitudinal section through tail vertebrae from a warfarin treated 6-week rat stained by von Kossa's silver technique to demonstrate calcium.
The epiphyseal growth plate (ep) shows extensive staining. d, vertebral disk. (x60)

d Enlargement of a similar section as seen in c. from a 6 week warfarin treated rat. There are many dark stained structures spanning the width of the epiphyseal growth plate (ep). (x300)


d
ep
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3.5 DISCUSSION

The results of this study show that warfarin-induced, extrahepatic vitamin K deficiency in the neonatal rat, causes differential growth retardation of the developing skull, resulting in maxillonasal hypoplasia. The presence of ectopic calcium deposits in the epiphyseal growth plates and in the septal cartilage of the nasal septum of the warfarin-treated rats indicates a generalised disturbance in the maintenance of uncalcified cartilage. It is not clear whether calcification of the septal cartilage is the cause of the maxillonasal hypoplasia, by reducing the longitudinal growth of the septum, or if they are both the result of another, more fundamental, disturbance. The consequences of the pathology in the growth plates are unknown but the effects should be deleterious. Since growth of the rat was slightly reduced the calcium bridges may slow down but not prevent the longitudinal growth of the bones.

Although the maxillonasal hypoplasia induced by postnatal warfarin exposure in the rat is distinctive, it is not nearly as striking as the most severe of the reported cases of the human warfarin embryopathy (e.g. Becker et al., 1975). There are several reasons for this difference. First, the most warfarin-sensitive stages of rat facial development are probably the last few days of fetal life in combination with early postnatal development. The present results are from postnatal exposure only. Secondly, the characteristic protrusion of the human nose is related to the extent that the cartilaginous nasal septum protrudes beyond the bony aperture of the nasal cavity. Normally about 20% of the total length of the nasal septum extends beyond this opening. If the posterior location of the septum is considered fixed, then a 10% decrease in the overall length of the septum in the human would result in a 50% reduction in the protrusion of the nose. In the rat, the length of the snout is the same as the length of the nasal septum, hence a 10% reduction in the length of the septum, will cause a corresponding 10% reduction in the length of the snout. If this analysis is correct, then similar reductions in septal growth in the rat and the human will cause very different changes in facial appearance.

Biochemical pathogenesis of the warfarin embryopathy

The earliest proposals for the pathogenesis of the warfarin embryopathy were based on the main clinical effect of warfarin, namely the prolongation of the blood clotting time. Warfarin inhibits the recycling of vitamin K causing an effective vitamin K deficiency resulting in the formation of non-functional decarboxylated prothrombin and other clotting factors. It was suggested that the nasal hypoplasia and the stippling were caused by microhemorrhages in the immature cartilage with subsequent scarring and calcification (Shaul et al., 1975). Against this theory was the observation that the critical period of exposure to warfarin was 6-9 weeks of gestation, which was before coumarin sensitive clotting factors were present in the embryo (Hall et al., 1980). Additional negative evidence came from the examination of a 17-week warfarin-affected fetus (Barr and Burdi, 1976). Microscopic examination did not detect hemosiderin deposits in areas of disordered chondrogenesis.

A subsequent theory (Hall et al., 1980) was based on the discovery that warfarin could interfere with the formation of another vitamin K-dependent protein; bone gla protein (BGP) (Price et al., 1976). This protein is secreted by osteoblasts and is normally bound to hydroxyapatite in the extracellular bone matrix. In the absence of vitamin K, BGP remains in a decarboxylated form and has a low affinity for hydroxyapatite crystals (Price et al., 1982). It was suggested that decarboxylated BGP, caused by warfarin therapy, might cause disordered ossification in the embryo (Hall et al., 1980). Against this theory is the observation that BGP is not present in cartilage (Luo et al., 1996) and that genetically-manipulated osteocalcin-deficient mice do not have skeletal-patterning defects or ectopic bone formation (Ducy et al., 1996).

In 1985 a second vitamin K-dependent skeletal protein - matrix gla protein (MGP) was discovered (Price and Williamson, 1985) and was shown to be present in cartilage (Hale et al., 1988). This has led to the suggestion that MGP is involved in the prevention of calcification in cartilage and hence in the warfarin embryopathy (Cole and Hanley, 1990).

In the present study, the concurrent administration of warfarin and vitamin K1 allows normal carboxylation of the vitamin K-dependent

proteins formed by the liver. These are the blood clotting proteins, notably prothrombin. None of the treated rats showed any evidence of hemorrhage and this can be excluded as a possible cause of the nasal hypoplasia or the ectopic calcification in the septal cartilage. In contrast, BGP and MGP are both formed extrahepatically and are likely to be in the decarboxylated state, because in the presence of warfarin, the extrahepatic tissues are unable to utilise excess vitamin K.

Although the precise role of MGP is unknown its presence in cartilage and its calcium binding gla residues suggest that decarboxylated MGP is involved in the process of the ectopic calcification in the septal cartilage and the calcium bridges in the growth plates.

Associated congenital abnormalities of the warfarin embryopathy

Schardein (1993) lists 25 cases of warfarin embryopathy with other malformations. Mostly these are CNS malformation that do not appear to be caused by hemorrhage. It would appear that the malformations that are not caused by cartilage hypoplasia in the first trimester or by hemorrhage in the second and third trimesters are presumably due to chance. The failure of the animal models (chapters 2 and 3) to produce any other malformations strongly suggests that warfarin does not produce malformations other than those of the warfarin embryopathy or those due to hemorrhage.

There is a report associating the Dandy-Walker malformation, agenesis of the corpus callosum and Peters anomaly with exposure to warfarin between weeks 8 and 12 of gestation (Kaplan, 1985). These malformations could be due to hemorrhage but blood clotting factors have not developed in the fetus by 12 weeks gestation.

If this association is not due to chance then a possible explanation would be that vitamin K levels once reduced remained low into the second and third trimesters even after the discontinuation of warfarin therapy resulting in hemorrhage. The neonate exhibited no other signs of warfarin embryopathy and there was no mention of vitamin K supplementation.

Summary and proposed morphological pathogenesis of the warfarin embryopathy

Schardein (1985) stated that coumarin anticoagulants were the only human teratogens that do not show teratogenicity in laboratory animals. The present study, in conjunction with the results in the previous chapter demonstrate in the rat most of the features of warfarin teratogenicity. Some peculiarities are evident because the sensitive periods are reversed. Facial dysmorphogenesis occurs following first trimester exposure to warfarin in the human while hemorrhage in the CNS occurs following second and third trimester exposure. In the rat, facial dysmorphogenesis occurs after perinatal exposure while CNS hemorrhage occurs after late prenatal exposure. Not all of the features of the warfarin embryopathy are seen in the rat, namely axial and long bone stippling, but there are sufficient similarities, including ectopic calcification in the growth plates, for this to be a good model to examine the effects of vitamin K deficiency on development.

CHAPTER 4

4.1 INTRODUCTION

Stippling or puncta are terms used to describe the radiographic image of abnormal calcification which appear as splatters or stipples within cartilage. They are present at birth in the cartilage in the ends of bone (epiphyses) and in the cartilage of the bones in the vertebral column in a variety of conditions including the warfarin embryopathy, the various forms of chondrodysplasia punctata, trisomy 18, trisomy 21, hyperparathyroidism, spondylo-epiphyseal dysplasia and acrodysostosis (Viljoen and Beighton, 1991; Poznanski, 1994).

In the warfarin embryopathy stippling is seen most commonly in the epiphyses of the axial skeleton (vertebrae and pelvis), proximal femora and calcanei (Hall et al., 1980). However, it can potentially occur in any cartilaginous area and in published warfarin cases stippling has been observed in the epiphyses of all of the long bones, scapulae, distal phalanges of the hand, tarsal bones, distal phalanges of the feet, cartilages of the ribs, and the laryngeal/tracheal cartilages (Pauli et al., 1976; Abbott et al., 1977; Robinson et al., 1978). Premature calcification of the hyoid bone has also been described (Becker et al., 1975). Although epiphyseal stippling is not a feature unique to the warfarin embryopathy it is a relatively common feature and it might be expected that it would occur in an animal model of the warfarin embryopathy.

The rat fetuses and pups treated with warfarin in the pre- and postnatal studies described in chapters 2 and 3 did not show typical epiphyseal stippling despite showing CNS hemorrhage and nasal hypoplasia. It is possible that neither the prenatal nor the postnatal warfarin treatments on their own cover enough of the critical period of rat bone development for stippling to appear.

Ossification begins on embryonic day 17 in the rat and is largely complete by 21 postnatal days. Hence the critical period for the induction of stippling may be late fetal and early postnatal life combined. Since stippling in the human is incorporated into completed bone and therefore

disappears by the completion of ossification (Theander and Pettersson 1978; Becker 1975; Hall et al., 1980) it is likely that the same is true for the rat. Therefore if combined pre- and postnatal treatment in the rat produced stippling it would only be detectable in the epiphyses before postnatal day 21.

As discussed in chapter 2 most prenatally warfarin-treated rat pups did not survive birth even at doses of 1 mg/kg/day of warfarin and 10 mg/kg/day of vitamin K which were not associated with obvious fetal hemorrhage. This dose had previously been considered the no-effect dose since hemorrhage was not detected in the fetuses of treated dams. However, when similarly treated dams were allowed to litter all the pups died and appeared bruised and hemorrhagic. This suggests that vitamin K-dependent blood clotting factors were depressed in the fetuses at this dose with the deficiency becoming evident with the trauma of birth.

It has been demonstrated that NADH-dependant reductase (necessary for utilisation of excess vitamin K1 to overcome the effects of warfarin) is present in the embryonic day 17 rat liver but at a low level. Levels of this enzyme rapidly increase after birth and adult levels were achieved by postnatal day 7 (Wallin, 1989). Hence it is possible that at 1 mg/kg/day warfarin and vitamin K1 10mg/kg/day the rat fetus is unable to utilise enough of the excess vitamin K1 to produce sufficient carboxylated blood clotting factors to prevent hemorrhage during birth. Concurrent administration of vitamin K up to 1000 mg/kg/day was unable to decrease mortality.

Further experiments were performed to determine the highest maternal intake of warfarin that in conjunction with vitamin K would be compatible with neonatal life. The established dose was 200 µg/kg/day warfarin combined with 10 mg/kg/day vitamin K1. This dose could be administered throughout prenatal life and most pups survived the birth process. If this dose of warfarin is administered without vitamin K the dams hemorrhage and die.

With these preliminary results in mind pregnant rats were treated throughout pregnancy with the non-lethal dose of 200 µg/kg/day of warfarin combined with 10 mg/kg/day vitamin K1 and after birth the

pups were treated daily until 16 days postnatally with 100 mg/kg/day of warfarin and 10 mg/kg/day vitamin K1. During the postnatal period the rats pups were killed and examined for evidence of stippling.

4.2 MATERIALS AND METHODS

Sprague-Dawley rats obtained from Castle Hill Animal House, University of Sydney were mated overnight and examined the next morning by vaginal smear. Rats with a sperm-positive smear were separated. The rats were given rat and mouse cubes (Y.S.F. Pty Ltd, N.S.W.) and tap water ad libitum and maintained under controlled conditions.

Seven pregnant rats were treated with 200µg/kg/day warfarin and 10mg/kg/day vitamin K1 from gestational day 1 to birth (prenatal day 22). The surviving pups from 4 litters were given a daily subcutaneous injection of 100 mg/kg sodium warfarin and 10 mg/kg vitamin K1 from postnatal day 1 to postnatal day 16. The lactating mother's of the treated litters were given daily subcutaneous injections of vitamin K1 to counteract any warfarin they ingested by their contact with the pups.

Litters from two untreated pregnant rats were used as controls. Three rat pups from each surviving treated litter were killed on days 12, 13, 14 and 15 and the remaining 3 rats from 3 litters were killed on day 16. Two control rat pups were killed on days 10, 11, 12, 13, 14 , 15 , 16, 17 , 18, 19 and 20.

4.2.1 Histology

The rat pups were skinned and eviscerated and the skeletons were stained with alizarin red (Kimmel and Trammel, 1981). The cleared skeletons were examined for abnormal calcification and any abnormalities were photographed.

4.3 RESULTS

Of the 7 treated pregnant rats 51 pups from 4 litters survived and were treated postnatally. The other 3 litters died shortly after birth due to haemorrhage. No deaths occurred during the postnatal treatment. The treated pups were healthy but were visibly smaller than their age-matched controls. Ossification appeared to be delayed by 2 or 3 days in the treated pups hence they were compared with controls at a similar stage of ossification.

Twenty-seven of the treated pups (53%) showed some abnormality of calcification of the developing skeleton. Most of these pups showed several abnormalities and they occurred in all age groups. The changes included an extra ossification centre in the cartilaginous part of the calcaneus (16 pups) (Fig. 4.1a), a bridging link between the ossification centre for the olecranon and the shaft of the ulna (15 pups) (Fig. 4.1b), an extra centre at the distal end of the humerus (4 pups) (Fig. 4.1c), one or two extra centres in the vertebral column (6 pups). Two pups had irregular calcification in one of the digits (Fig. 4.2a), 2 had similar changes in the sternum (Fig. 4.2b). Thirty-four of the pups showed a small area of calcification in the nasal septum (Fig. 4.2c). This occurred mainly in the superior border underlying the nasal bones. No calcification was observed in the control nasal septa.

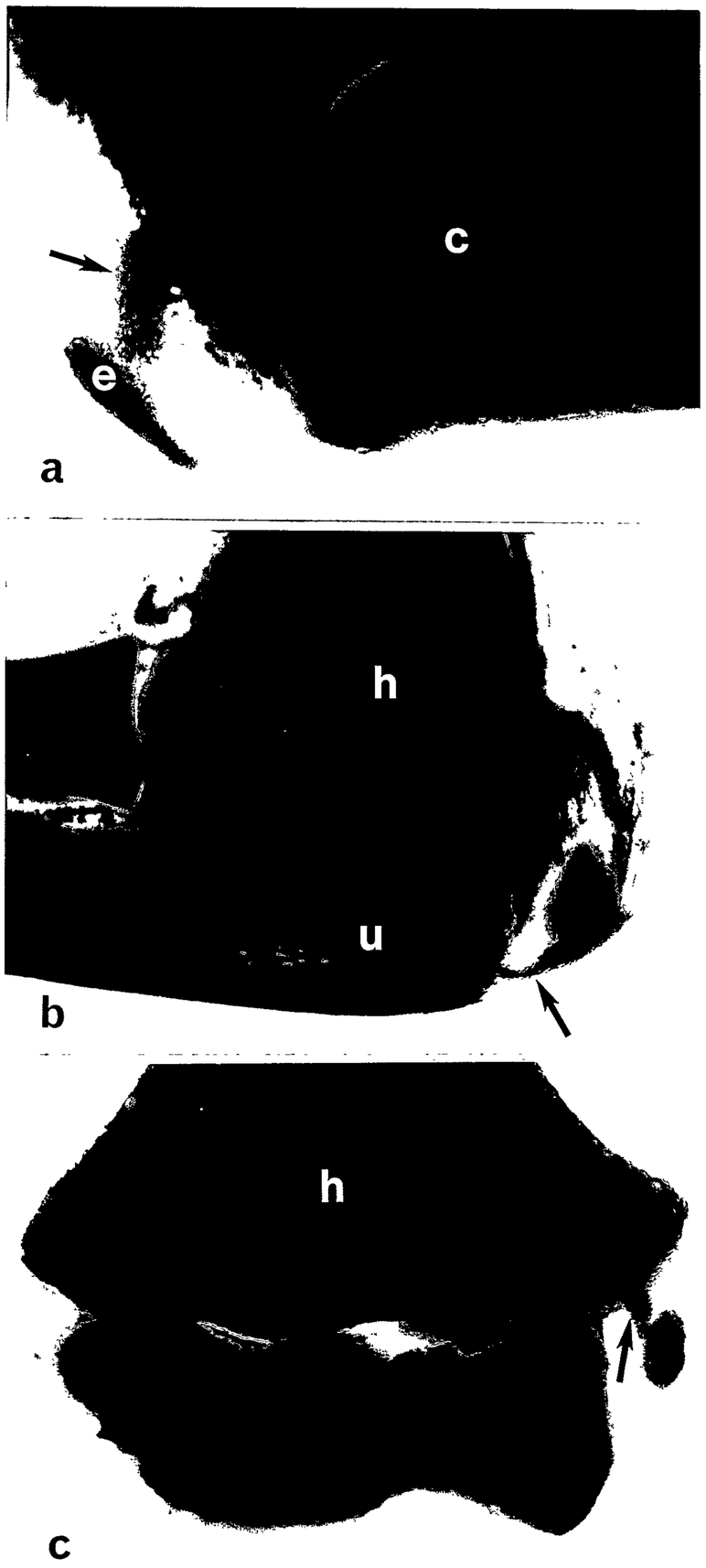
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Figure 4.1

a. Alizarin-stained hindlimb of a 14 day rat pup treated pre- and postnatally with warfarin. Note the area of ectopic calcification (arro in the cartilaginous part of the calcaneus. e = epiphysis

b. Alizarin-stained forelimb of a 14 day rat pup treated pre- and postnatally with warfarin. Note the bar of ectopic calcification (arrow passing from the epiphysis to the diaphysis of the ulna. h = humeru u = ulna

c. Alizarin-stained forelimb of a 14 day rat pup treated pre- and postnatally with warfarin. Note the area of ectopic calcification (arrov next to the normal ossification centre for the epicondyle. h = humeri

Figure 4.2

a Alizarin-stained forelimb of a 12 day rat pup treated pre- and postnatally with warfarin. Note the ectopic calcification (arrow) at the proximal end of the distal phalanx. p = distal phalanx

b Alizarin-stained sternum of a 14 day rat pup treated pre- and postnatally with warfarin. Note the ectopic area of calcification extending from the xiphoid process (arrow). s = sternum x = xiphoid process

c Alizarin-stained nasal septum from a day 16 rat pup treated pre- and postnatally with warfarin. Note the area of ectopic calcification in the superior border of the septum (arrows) below the position of the nasal bones. n= nasal septum e = perpendicular plate of the ethmoid bone

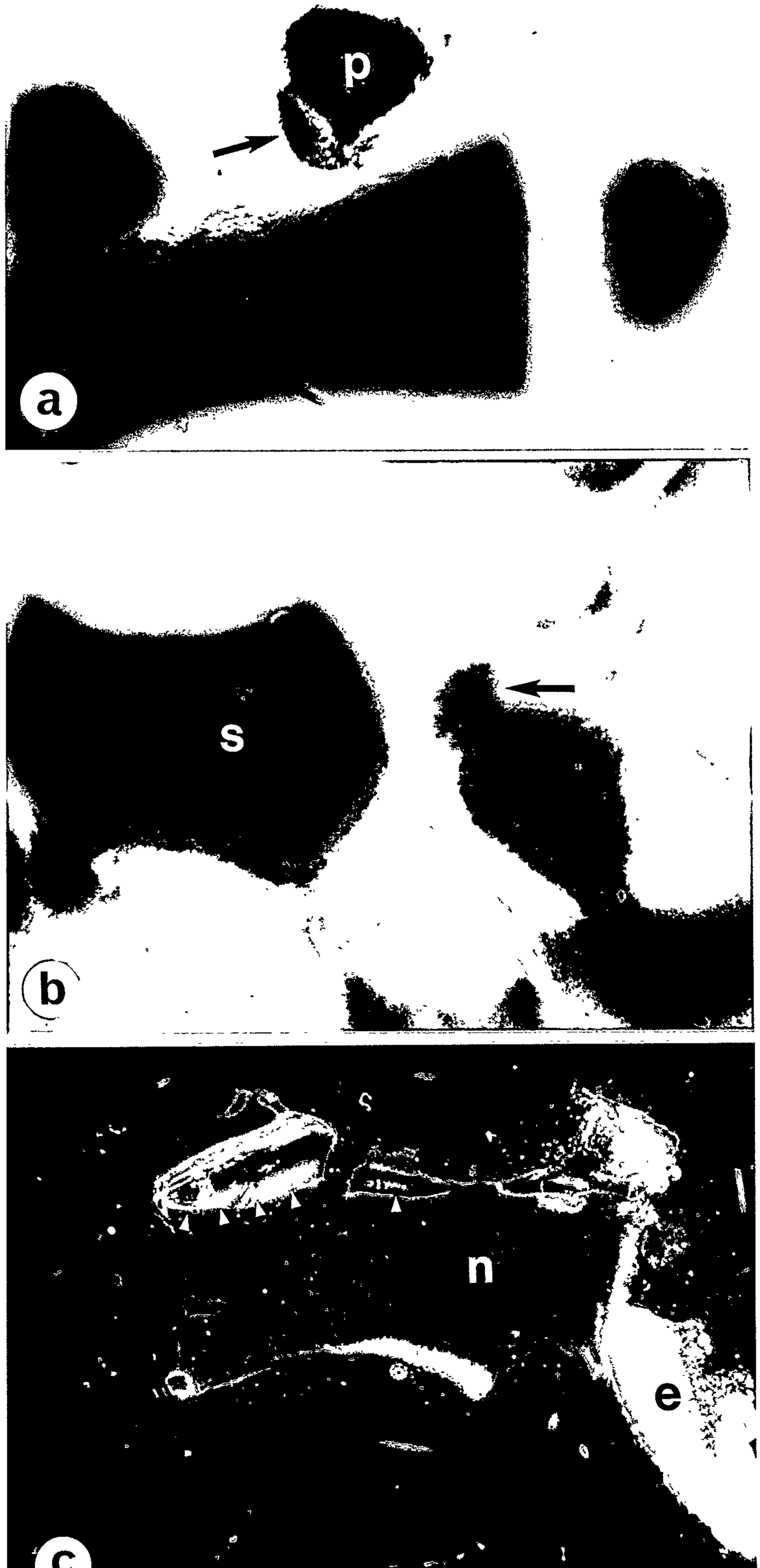
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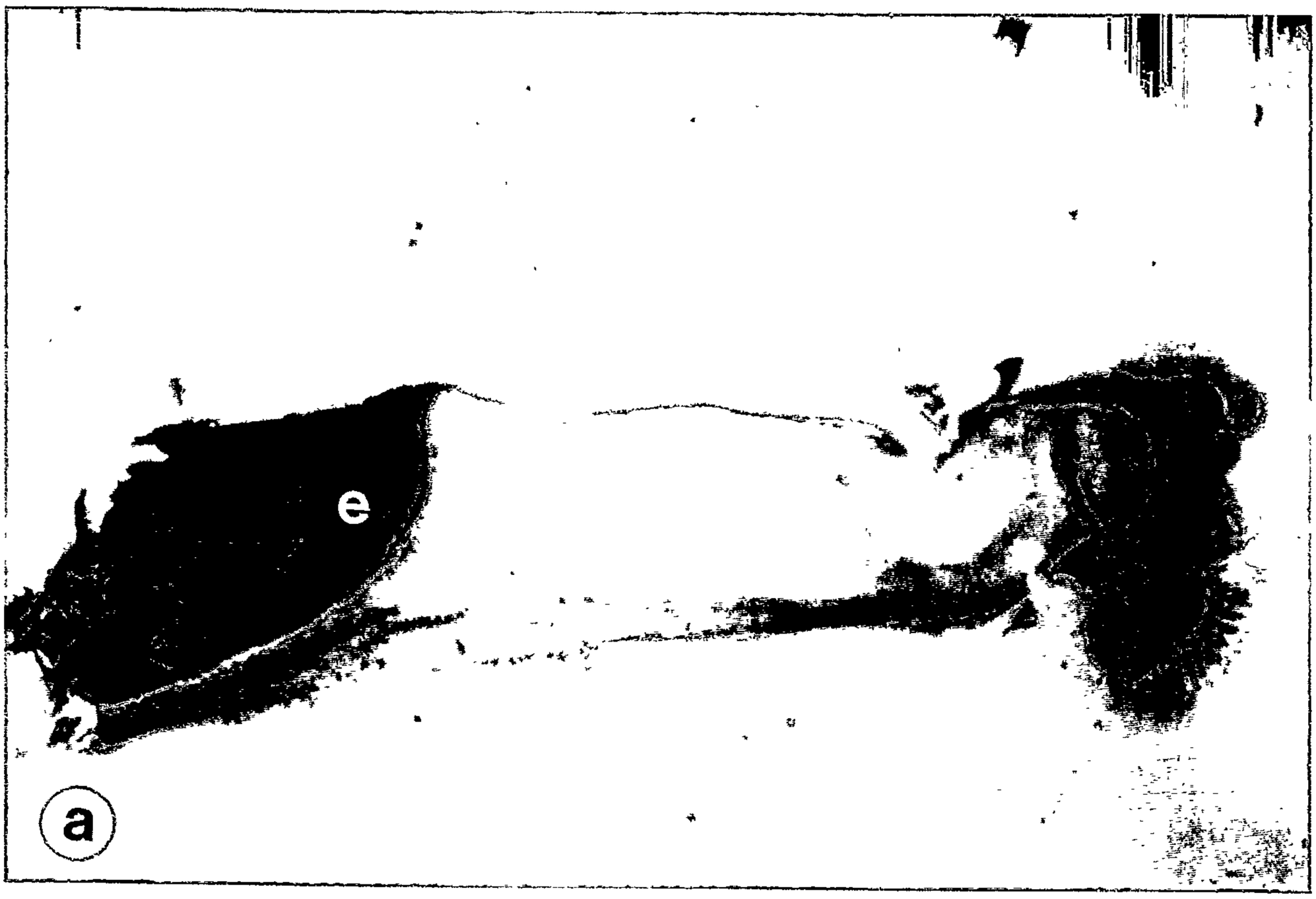
Figure 5.1

a Alizarin stained 12-week control (non treated) nasal septum. The perpendicular plate of the ethmoid bone (e) stains red due to the presence of calcium. The slight red staining along the lower border of the otherwise unstained nasal septum is an artefact from the position of the bony vomer left in place during processing. Note the absence of calcium in the cartilaginous nasal septum.

b Alizarin stained 12-week control (non treated) nasal septum. The perpendicular plate of the ethmoid bone (e) and the vomer (v) stain red due to the presence of calcium.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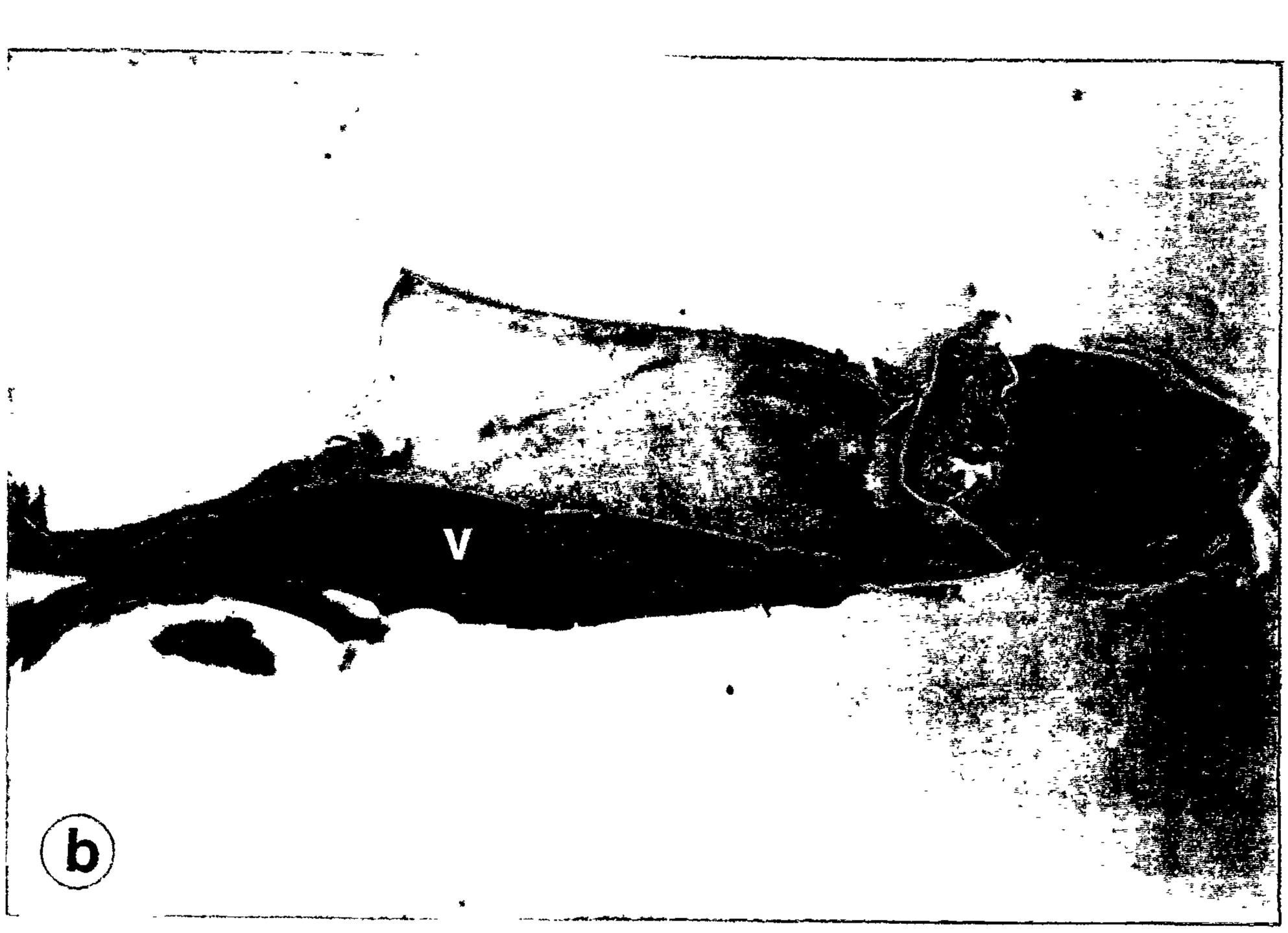
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Figure 5.2

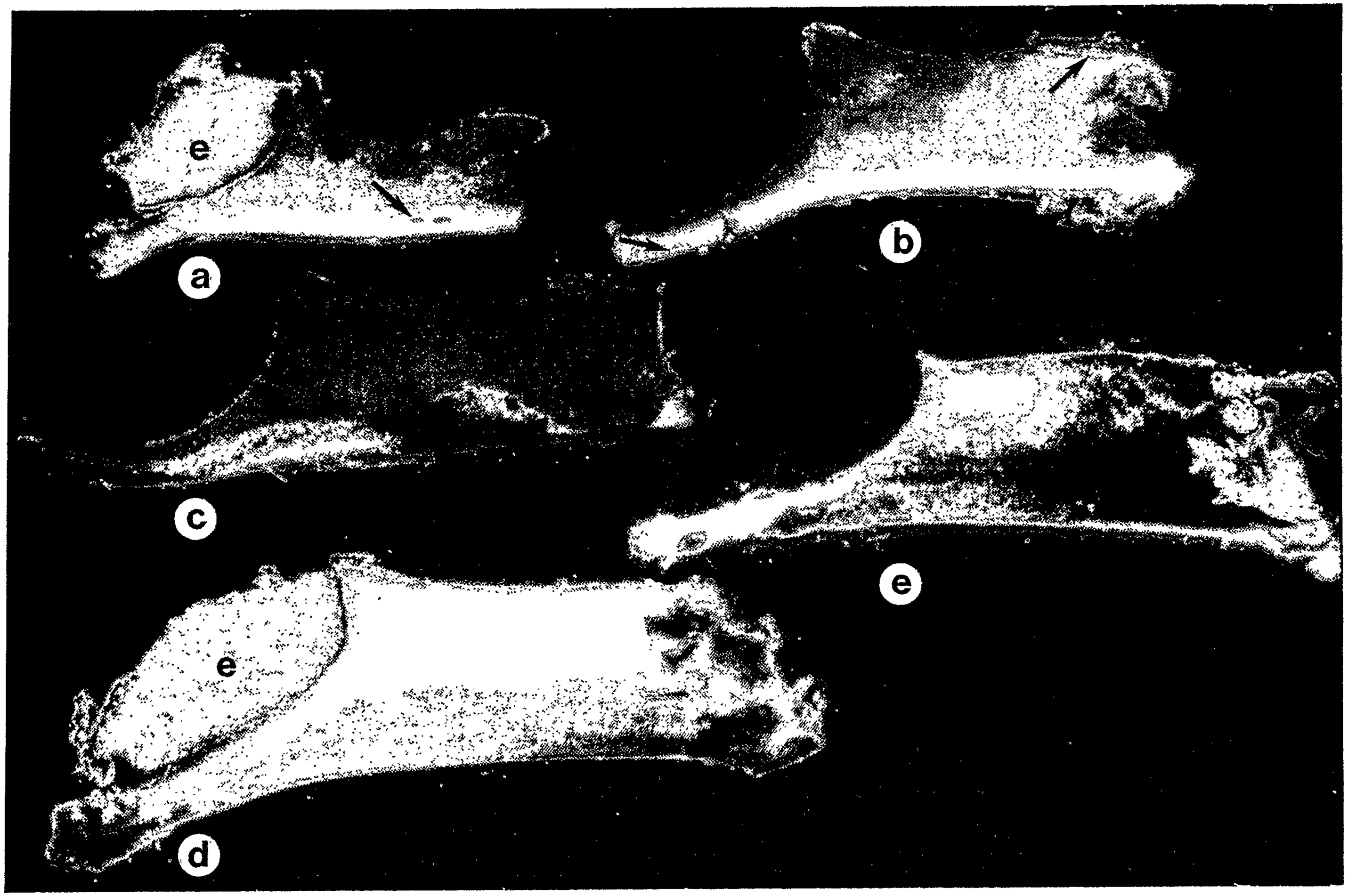
a Alizarin stained nasal septum from a 3-week warfarin treated rat. Arrows indicate the presence of red stained calcium deposits.

b Alizarin stained nasal septum from a 5-week warfarin treated rat. Arrows indicate the presence of red stained calcium deposits.

c Alizarin stained nasal septum from a 7-week warfarin treated rat. Note the presence of red stained calcium deposits.

d Alizarin stained nasal septum from a 9-week warfarin treated rat. Note the presence of extensive red stained calcium deposits.

e Alizarin stained nasal septum from a 12-week warfarin treated rat. Note the presence of extensive red stained calcium deposits.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4.4 DISCUSSION

Although many of the warfarin-treated rat pups showed one or two abnormalities of calcification in the cartilaginous ends of bones, they did not show the multiple centres of calcification usually depicted in the warfarin embryopathy. This may be related to the greater bulk of cartilage at the ends of bones in the developing human. If stippling is considered a disturbance in the normal development of cartilage it may be more likely to occur in large pieces of cartilage which have greater problems with diffusion of nutrients. It is noteworthy that stippling in the human is usually described in the largest cartilages eg. the proximal femur and calcaneus. If volume of the cartilage is an important parameter in the development of stippling it is not surprising that stippling is not readily induced in the rat.

Another parameter that varies considerably between the rat and human is the prolonged period that the developing human skeleton has cartilaginous ends to the bones and hence is exposed to the effects of warfarin. For instance, the calcaneus first appears as a cartilaginous structure at about 6 weeks gestation and is still mostly cartilaginous at birth. Hence the cartilaginous parts of the human skeleton are exposed to warfarin-induced vitamin K deficiency for about 31 weeks while the combined pre- and postnatal treatments in the rat expose the cartilaginous ends of the bones to vitamin K deficiency for about 15 days. In contrast, most of the rat nasal septum remains cartilaginous throughout the animal's life and ectopic calcification is readily induced. With postnatal warfarin-treatment calcification first appears in the septum after about 3 weeks treatment. The calcification continues to increase up to 12 weeks treatment when most of the normally cartilaginous part of the septum is calcified. This indicates that ossification in the skeleton may be too rapid in the rat for generalised stippling to appear.

A review of 418 published cases of warfarin exposure during pregnancy concluded that the warfarin embryopathy, of which stippled epiphyses is a variable characteristic, was not observable unless exposure was in the first trimester (Hall et al., 1980). There are 27 well-documented cases that show both the facial features of warfarin embryopathy and stippling (Schardein, 1993). In 18 cases warfarin exposure occurred in all three trimesters. In

five cases warfarin exposure only occurred in the first two trimesters and in four cases only in the first trimester.

This data seems to supports the contention (Hall et al., 1980) that stippling occurs only after first trimester exposure to warfarin. There are no reports of stippling after warfarin exposure confined to the second or third trimesters only. However, the number of cases with appropriate exposure and neonatal radiographs is small (Sherman and Hall, 1976; Chong et al., 1984; Kaplan 1985; Wong et al., 1993). It is possible that abnormal calcification can be induced by warfarin treatment at any time during pregnancy since there are several reports that children on long term postnatal warfarin therapy exhibit ectopic calcification in the growing cartilages of the trachea and larynx (Rifkin and Pritzker, 1984; Taybi and Capitanio, 1990).

It is important to note that the facial dysmorphogenesis can occur without stippling. In a review of published cases, all of which had nasal hypoplasia, stippling occurred in 88% of cases where appropriate radiographs were taken (Pauli, 1997). In a prospective study of 27 pregnancies that resulted in live births and were exposed to warfarin throughout the pregnancy, warfarin embryopathy occurred in 8 of the offspring but they did not have stippling (Iturbe-Alessio et al. 1986). Similarly in another series (Wong et al., 1993) there were 17 cases with nasal hypoplasia yet none of the cases exhibited stippling despite neonatal radiography. This would imply that mechanisms influencing nasal septum growth are more sensitive to warfarin exposure than are the mechanisms influencing the development of stippling.

The histology of stippling in the warfarin embryopathy.

There is a description of the histology of stippling in an infant with the warfarin embryopathy who died shortly after birth (Becker et al. 1975). There were well-delineated foci of atypical cartilage in the epiphyseal regions of the tarsal bones, upper part of the tibia, and the head of the femur. These foci consisted either of areas of proliferation of small chondrocytes associated with calcification of the matrix or of regions of acellular myxomatous material and numerous thin walled vascular channels. Focal penetration of the epiphyses by periosteal fibrovascular tissue was seen just beyond the cartilaginous columns of the zone of

endochondral ossification. One area of fibrosis and increased vascularity was found to interrupt the proximal tibial metaphysis. Radiologically there was evidence of stippling.

Similarly in a report of warfarin embryopathy in a 17-week abortus which displayed facial flattening and digital hypoplasia. At autopsy there was abnormal proliferating cartilage especially in the distal phalanx. There were also irregular masses of chondrocytes producing thick, nodular and bulky epiphyses. On distal phalanges 1-4 there were aberrant clumps of chondrocytes connecting the shaft with the osteoid tuft. Barr and Burdi concluded that stippling was a result of a basic disorder of chondrogenesis. At 17 weeks there was no calcification only cartilage changes (Barr and Burdi 1976). This may suggest that disorganisation of chondrogenesis proceeds calcification.

The Effects of Stippling.

Stippled or punctate epiphyses in long bones are not usually considered a serious problem as they are incorporated into the developing bone in the postnatal period. However, stippling in the growth plate may be of greater consequence and may be responsible for the growth retardation and abnormal bone development associated with stippling in some conditions (Poznanski, 1994). The outcome may be related to the severity of the stippling. It is assumed that stippling represents premature calcification in the cartilaginous ends and/or growth plates of the bone and like the calcification in the nasal septum is a consequence of abnormal MGP. Decreased growth of bones may be expected with stippling in growth plates and some cases of rhizomelia have been reported in children with the warfarin embryopathy (Becker et al., 1975; Raivio et al, 1977; Whitfield 1980; Hosenfeld and Wiedemann, 1989). A recent literature review indicates limb abnormalities such as digital hypoplasia and/or foreshortening in 59% of published cases (Pauli, 1997). Similarly calcification of cartilaginous structures such as the nasal septum (Howe and Webster 1992) or trachea can lead to decreased growth of these structures (Kaufmann et al., 1967). The consequence of stippling in the vertebrae may also be underdevelopment and in some cases abnormal development such as scoliosis (Hall et al., 1980).

Is the ectopic calcification seen in the rat the same as human stippling?

The rat does not exhibit the same manifestations of ectopic calcification as seen in the human warfarin embryopathy. The absence of stippling appears to be related to duration of bone development and perhaps bulk of cartilage. In structures that remain cartilaginous for extended periods of time in the rat, such as the growth plates and nasal septal cartilage, long term warfarin exposure causes extensive ectopic calcification. Similar changes in the growth plate have not been reported in the human warfarin embryopathy because the growth plates do not appear prenatally.

CHAPTER 5

5.1 INTRODUCTION

It was shown in chapter 3 that rats treated postnatally with warfarin had substantial areas of ectopic calcification in their nasal septal cartilage when viewed at 12 weeks. The distribution of the calcification was similar in all the treated rats which suggested that certain parts of the septum were more likely to calcify than others.

It was also observed in chapter 3 that rats treated from birth with warfarin had a clearly hypoplastic snout after only 3 weeks treatment. If calcification of the nasal septum was the cause of the hypoplasia it would be expected that a significant amount of the cartilage would be calcified by that age.

To examine this hypothesis neonatal rats were treated with warfarin from birth and killed at 3 weeks. Both external nasal length and internal nasal septal length were measured. Other rats were similarly treated and killed at various intervals up to 9 postnatal weeks and the nasal septal cartilage removed and its calcium content examined qualitatively and quantitatively.

5.2 MATERIALS AND METHODS

Sprague-Dawley rats obtained from Castle Hill Animal House, University of Sydney were mated overnight and examined the next morning by vaginal smear. Rats with a sperm-positive smear were separated and allowed to litter. The day of birth was considered postnatal day 0. The rats were given rat and mouse cubes (Y.S.F. Pty Ltd, N.S.W.) and tap water ad libitum and maintained under controlled conditions. On postnatal day 1 each litter was culled to 10 pups and randomly assigned to one of four experimental groups.

Group 1 consisted of 2 litters in which the pups were given a daily subcutaneous injection of 100 mg/kg sodium warfarin (supplied and assayed by Boots Company, North Rocks, Sydney) dissolved in distilled water at 100 mg/ml. At the same time the pups were given a daily subcutaneous injection of 10 mg/kg vitamin K1 (Konakione, Roche Pharmaceuticals). The litters were killed at 3 weeks and each rat pup was sexed and weighed. Body length, tail length and snout length (inner canthus to tip of nose) were measured to the nearest 0.5 mm. The skull was dissected to display the sagittal midline and the length of the nasal septal cartilage (superior border from the distal end of the perpendicular plate of the ethmoid to the nasal tip) was measured *in situ* to overcome the shrinkage that occurs after it is removed from its bony attachments.

Group 2 were controls for group 1 and consisted of two untreated litters that were killed at 3 weeks and measured as described for group 1.

Group 3 consisted of 2 groups of 2 litters that were treated with warfarin and vitamin K from postnatal day 1 as described in group 1. Treatment continued for up to 9 weeks. Four rats from 2 litters were killed at 2, 3, 5, 7 or 9 weeks. It was originally intended that only two litters would be needed but pup deaths (maternal irritability!) necessitated a second series of treatments. The nasal septal cartilage from each rat was removed and either stained with alizarin red (Kimmel and Trammel, 1981) and photographed or analysed for calcium content. For calcium measurement the nasal septa from the 2 - 7 weeks of treatment were trimmed under magnification to remove all obvious calcification from the margins of the cartilage. The trimmed septum was weighed and dissolved in 1 ml of

15.8 N nitric acid. This solution was then analysed for calcium content using atomic absorption spectrophotometry. The results expressed as mg calcium per gram wet weight of sample. For the 9 week cartilages the large calcified area was also analysed.

Group 4 was controls for group 3 and consisted of two untreated litters that were killed and examined as described for group 3.

Statistics The results of the growth measurements taken at 3 weeks were analysed by student's t-test, unpaired. The results of the calcium analysis were not analysed statistically because there were only two septa for each value.

5.3 RESULTS

Growth measurements at 3 weeks.

When the treated and control rats were separated into males and females and measured at 3 weeks there were no significant differences between the two groups for body or tail length or body weight (Table 5.1). External nose length was reduced significantly by 10.2% in the treated males and 8.8% in treated females. The external nose length corresponded well with the length of the nasal septum.

Table 5.1 Measurements of control and warfarin-treated 3-week old rats

	No. of rats	body length (mm±SD)	tail length (mm±SD)	body weight (g.±SD)	ext nose length (mm±SD)	nasal septum length (mm±SD)
control male	9	212.8±7.0	78.2±3.4	70.8±5.0	16.6±0.5	16.6±0.6
control female	8	203.8±8.1	77.4±4.6	66.9±7.0	16.0±0.8	16.4±0.7
treated male	8	214.8±8.6	81.9±4.8	70.9±6.2	14.9±0.5[1]	14.9±0.6[1]
treated female	6	212.5±8.3	81.8±5.6	67.3±8.1	14.6±0.7[2]	14.5±0.8[1]

[1] significantly differently from control group $P < 0.0005$

[2] significantly differently from control group $P < 0.005$

Calcification of the nasal septa - alizarin-staining

The normal nasal septal cartilage, as seen in the untreated controls, did not show any alizarin staining when examined between 2 and 9 weeks postnatally. It was always stained with the adjacent perpendicular plate of the ethmoid which acted as a positive control area (Fig. a and b).

After only two weeks treatment the nasal septal cartilage from the warfarin-treated rats did not show any calcification but with longer periods of treatment areas of ectopic calcification were apparent. Septa from 2 rats were examined after 3 weeks treatment and both showed a small area of calcification in the antero-superior border and one showed 2 additional areas in the inferior border (Fig. 5.2a). After 5 and 7 weeks treatment further calcification was evident along the border of the nasal septum that

abutted the nasal bones and in an area in the inferior border abutting the vomer (Fig 5.2 b and c). Small areas were also observed at the junction with the ethmoid. After 9 weeks treatment the calcified areas seen in the 5 and 7 week septa had increased and a substantial area was calcified along the lower border abutting the vomer (Fig. 5.2d). For comparison a picture of an alizarin-stained nasal septum after 12 weeks warfarin-treatment is included (Fig 5.2e). This animal was part of the experiment described in chapter 3.

Calcification of the nasal septa - Atomic absorption spectrophotometry

To determine whether warfarin-treatment caused a progressive increase in the calcium content of the nasal septal cartilage, the calcium content was measured after 2, 3, 5, 7 and 9 weeks of warfarin treatment (Table 5.2). Visibly calcified areas were removed so only apparently normal cartilage was analysed. Although there was some variation in the calcium content with age, with one exception, there were no significant differences between treated and untreated cartilages. The exception was the treated cartilages at 5 weeks which had a much higher calcium level than the controls and a much higher level than the 7 and 9 weeks treated septa. The figure of 0.988 consisted of values from 2 septa (0.461 and 1.515 mg/g). It is assumed the higher figure relates to an isolated piece of calcium in the nasal septum which was missed at dissection (eg. Fig. 5.2b). A piece of calcified nasal septum removed from a 9-week treated rat and analysed separately had a calcium content of 22.70 ± 5.68 mg/g wet tissue.

Table 5.2 Calcium content of nasal septa

Age of sample	Control nasal septa calcium mg/g ± SD (number of septa)	Treatment nasal septa calcium mg/g ± SD (number of septa)
2 weeks	0.175± 0.019 (2)	0.213± 0.004 (2)
3 weeks	0.121± 0.001 (2)	0.148± 0.014 (2)
5 weeks	0.381± 0.018 (2)	0.988± 0.745 (2)
7 weeks	0.354± 0.018 (2)	0.356± 0.016 (2)
9 weeks	0.322± 0.0.03 (2)	0.325± 0.025 (2)

5.4 DISCUSSION

After 3 weeks warfarin-treatment there was a significant difference in the nasal septal length between the treated and control rats. However there was only minimal ectopic calcification in the septum at this age as shown by alizarin-staining. Measurements of total calcium in the remaining cartilage showed that there was no increase in calcium content in this tissue. These results suggests that the retardation of septal growth is not due primarily to calcification of the septal cartilage.

Growth of the nasal septum

The cartilaginous nasal septum is made up primarily of two components; cells (chondrocytes) and intercellular matrix, the latter making up the greater part of the bulk of the septum (Searls, 1979). The chondrocytes are actively dividing in the postnatal period and growth of the septum is believed to be dependent upon the synthesis by these cells of intercellular matrix (Searls, 1979). Areas with the greatest concentrations of proliferating cells are also likely to be the sites of growth since chondrocyte proliferation is followed by increased matrix formation (Kvinnsland, 1977). In a study of 5-day rat septa it was concluded that the septoethmoidal junction was a primary growth site and that the septal cartilage was thrust forward from the perpendicular plate of the ethmoid (Searls, 1975). In contrast Copray (1986) measured growth pressures in the 4-day-old rat nasal septum in vitro and concluded that the thick rod-like base is the structure where the greatest growth force can be developed.

Matrix gla protein (MGP)

The mechanism by which warfarin-treatment causes reduced growth of the septal cartilage is assumed to be related to the vitamin K-dependent MGP. MGP is secreted by a wide range of tissues including lung, heart, cartilage and kidney but it only accumulates in the matrix of bone, cartilage and dentine (Hale et al., 1988; Fraser and Price, 1988). Chondrocytes have been shown to secrete MGP (Barone et al., 1991; Luo et al., 1995) and it accumulates in the cartilage as a structural protein bound to the organic matrix (Price et al., 1983). The gla residues on MGP confer a high affinity for calcium, phosphate ions and hydroxyapatite crystals

(Mallein-Gerin et al., 1988) and it may inhibit hydroxyapatite formation (Hale et al., 1988). These mineral binding properties suggest that the protein plays a role in the control of mineralisation in the development of bone and cartilage (Luo et al., 1995).

In a recent *in situ* hybridisation study in the mouse embryo it was shown that MGP mRNA is predominantly expressed in cells of the chondrocytic lineage both in areas that will subsequently ossify and in areas such as the trachea and bronchi that will remain cartilaginous (Luo et al., 1995). In growth plate cartilage, MGP mRNA is present in resting, proliferative, and late hypertrophic chondrocytes but is absent from the early hypertrophic chondroblasts and osteoblasts.

The ability of MGP to bind hydroxyapatite, its presence in cartilage and the abnormal calcification of cartilage seen with warfrain treatment all suggest that MGP plays a role preventing calcification. However, as pointed out by Price (1989) since warfarin does not cause calcification of soft tissue, where MGP is expressed but does not accumulate, it is unlikely that MGP functions exclusively as a calcification inhibitor.

Intracellular calcium has been suggested as a regulator of chondrogenesis. Several studies (Deshmukh et al., 1976, 1977; Maor et al., 1985) have suggested that chondrocytes cultured *in vitro* are modulated by calcium supplementation such that collagen type synthesis and mitotic rates are significantly altered. In addition, growth plate chondrocytes at various stages of their life cycle must also respond differently to graded changes in environmental calcium as they advance from the resting and proliferative stages to hypertrophy and mineralisation (Brighton, 1978); for example, recent data suggest that matrix vesicle biogenesis, a property of hypertrophic chondrocytes, may be regulated by calcium (Iannotti et al., 1989). These data suggest that chondrocytes are continuously influenced by their calcium milieu throughout their life cycle (Tuan, 1991) and this may involve MGP.

The ability of warfarin to reduce cartilage growth may be due to the presence of non-functional MGP in the cartilage interfering with the chondrocyte life cycle and reducing proliferation and/or extracellular matrix formation. The subsequent development of ectopic calcification

may be a further consequence of altered chondrocyte function. Irregular chondrogenesis has been previously described in the post-mortem examination of a human fetus and a newborn with the warfarin embryopathy (Becker et al., 1975; Barr and Burdi, 1976).

Interestingly the phenotype of warfarin embryopathy (nasal hypoplasia and stippling) is seen in a hereditary chondrodysplasia punctata which has been linked to a point mutation in the arylsulfatase E gene. The function of arylsulfatase remains unknown but it was suggested that it may be essential for correct composition of cartilage matrix during development (Franco et al., 1995). Abnormal matrix development may be the common link between this condition and the warfarin embryopathy. Any genetic condition or environmental agent that similarly interferes with cartilage matrix formation and/or chondrogenesis may produce this phenotype and may explain the seemingly disparate groups that make up the chondrodysplasia punctatas. If the matrix component occurs in other structures or processes in development other abnormalities may also be present.