Study of a mouse model for the oromandibulo-facial limb hypogenesis (OMFL) syndrome following chorionic villus sampling

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Submitted in satisfaction of the requirement for the degree of Ph.D. in the University of Edinburgh 1998

ABSTRACT

A mouse model has been established to investigate the teratogenic effects of amniotic sac puncture (ASP) performed during a similar period during gestation when chorionic villus sampling (CVS) would normally be carried out clinically. The abnormalities encountered in this model have many of the craniofacial and limb features observed in the oromandibulo-facial limb hypogenesis (OMFL) syndrome (also termed oromandibular-limb hypogenesis syndrome, OMLHS), in which, *limb* defects ranging from transverse limb reduction defects, through adactyly, syndactyly to nail hypoplasia (in the least affected cases), have caused concern clinically. While the abnormalities in the craniofacial region are believed to have a postural basis, the aetiology of the limb defects is unclear. Limb and other postcranial skeletal defects induced by ASP have been analyzed in detail after embryos have been double-stained with Alizarin red S and Alcian blue to reveal their bony and cartilaginous elements, respectively. The results revealed that even in cases of adactyly, all of the skeletal elements of the autopod were present; this implied that the syndactylism observed exclusively involved the soft tissues of the interdigital zones. Other findings revealed that the affected embryos were retarded in development. An additional striking finding was that the ossification pattern of sternebrae was also frequently grossly abnormal, though the cause has yet to be established.

Many hypotheses have been proposed regarding the underlying relationship between limb defects and CVS. Based on experimental and clinical evidence, much interest has been directed towards exploring the possibility of vascular disruption as the likely mechanism involved. In addition, it has been proposed that inadvertent rupture of the amnion might occasionally occur during CVS, leading to leakage of amniotic fluid and oligohydramnios. This might result in the compression of the embryo by the uterine muscles and extraembryonic membranes, leading to interference with the embryonic circulation and ischaemia and/or hypoperfusion of the distal extremities. Investigations of the embryonic heart rate in both control and experimental groups following ASP confirmed this possibility. Exposure to the anaesthetic Avertin alone produced embryonic bradycardia within the period of 30 minutes after injection. Similar findings were observed in embryos whose mothers had received Avertin and laparotomy but without being exposed to ASP. In embryos that had been subjected to ASP an even more prolonged period of bradycardia was observed. This suggested that the venous stasis commonly observed in the periphery of the digits might account for the association between cvs and limb defects.

Histological analysis of the interdigital spaces of both control and experimental autopods at intervals following ASP revealed that vascular disruption in the form of dilatation, congestion, haemorrhage and fluid-filled cavities, was commonly encountered. This was invariably noted in

association with the marginal vein and in the capillary networks between the digits of the experimental autopods as soon as 0.5h after ASP. The cell death program (apoptosis) in these areas also declined dramatically, while at the same time, the cellular mitotic activity in the interdigital zones increased after 4h following ASP. This suggested that vascular disruption probably interfered with the pre-set program which controls the cell fate in these areas. The underlying mechanism(s), however, remained to be established. It is hypothesised that it may involve the stimulation of tissue regeneration, possibly due to *overexpression* of certain gene(s), and/or growth factor(s) that are brought to the vicinity by macrophages during their clearing of the necrotic cells.

Many genes are expressed temporally and spatially in the interdigital zones during limb morphogenesis, and may play a key role of triggering the pre-set program within these areas. Of the latter, *Msx-1* is believed to maintain the proliferative and undifferentiated status of cells in the interdigital zones as well as being associated with the control of programmed cell death that occurs in these areas. Detection of the expression of *Msx-1* in the interdigital zones at intervals after ASP confirmed that this gene is upregulated in this area in the experimental autopods by 4h after ASP, and that this is accompanied by evidence of epithelial hypertrophy. This study demonstrated that *Msx-1* maintains the cells in the interdigital zones in a proliferative and undifferentiated state, as has been hypothesized by others, perhaps partly due to interference with epithelial-mesenchymal interaction.

When Halothane, instead of Avertin was used as the anaesthetic during ASP, we have speculated that this could not only dramatically *reduce* the recovery time, but also improve the embryonic/fetal circulation to the autopod after ASP. Although similar overall rates of abnormalities were observed, the incidence of *syndactyly* was highly significantly reduced. This supported our hypothesis that venous stasis along with vascular disruption in the distal extremities may abolish the normal pre-set programs in the interdigital zones and influence the type of limb defects observed.

In summary, if inadvertent rupture of the amnion occurs during the CVS procedure, fetal/embryonic venous stasis could occur as a secondary consequence of the compression of the embryo/fetus by uterine muscles and extraembryonic membranes. The induction of a similar range of limb defects in our mouse model to those observed clinically following cvs were investigated in this study. In addition, vascular disruption in the limbs following ASP was observed histologically, and it is suspected that this may be closely associated with the induction of syndactyly which was observed in 74% of the cases in this model. It has been hypothesized that after the vascular disruption, the epithelial-mesenchymal interaction may be distorted and

the dynamic equilibrium of the pre-set cell pattern program in the interdigital zones may also be interfered with. Accordingly, the over-expression of *Msx-1* in this area might induce tissue regeneration in the interdigital zone, as well as interference with apoptosis, leading to the high incidence of syndactyly seen in the Avertin model.

DECLARATION

This thesis does not contain without acknowledgement any material previously submitted for a degree or diploma in any university, and except due reference is made, does not contain any material previously published or written by any another person.

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ACKNOWLEDGMENT

I would like to say thank you to the following individuals --

First of all, I am most grateful and indebted to my major supervisor Professor Matthew H. Kaufman. He offered me an opportunity to enter the field of mammalian embryology and guided me throughout the entire course of this study with unfailing enthusiasm and dedication.

Special thanks are due to Dr. John D. West and Dr. Sarah E. Wedden for acting as my supervisors. I have received many useful discussions and supports from them.

I wish to thank Mr Nigel Bain and Mr Jack Cable for their expert photographic assistance.

My gratitude also goes to Mrs. June Gillam and Sheila Hunter for sharing with me their computer and printing facilities. Drs. Arthur Jurand, Jamie Davies, Jonathan Bard, James Shaw, Clare Gribbon all gave me many hours of their time. Dr. Gordon Findlater and Mr. Bill Adams assisted me with the statistics used in this study. Dr. James Shaw kindly instructed me to use the Kontron image analysis system required in chapter 5.

Much support, advice, and friendship was given by Bob, Nigel, Jane, Mark, Tom, Gary, Ronnie and Corinne. Without their help, it would not have been possible to complete this work.

Extra-special thanks are due to Donald MacIntyre, Zachary Schwartz, Yincent Tse and Julie Gordon for their technical assistance and support. Donald carried out the statistical analyses and took photographs presented in chapter 2 during his BSc (Med Sci Honours Anatomy project). Zachary involved in the preliminary studies reported in chapter 3 when he was supported by a Wellcome Trust Vacation Scholarship. Yincent and Julie provided me the conventional paraffin and semi-thin sections, respectively, for studies reported in chapter 5 and 6, respectively. Both of them were BSc Honours students in the Department of Anatomy. All of the project work undertaken by these students was supervised by Professor Kaufman.

Finally, I am very gratefulto my husband -- David, for his encouragement and supports and to my son -- Herbert who has always tried to cheer me up despite my constant distractions in other directions. In particular, many thanks must also go to my parents for their understanding and support throughout my studies.

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Chapter 1: Introduction

1.1 Methods of prenatal diagnosis

About 2% of live births display some kind of major abnormality (Thorogood, 1997), depending on the locality, culture, ethnic background and the efficiency of recognition and reporting. If minor abnormalities are included, then the incidence is nearer to 5 %. Congenital abnormalities constitute the greatest cause of infant mortality and have a major impact on national health budgets in Western countries (Sever *et al.*, 1993). Prenatal diagnosis, therefore, has become increasingly important in the last decade, and has certainly been one of the most interesting, controversial and rapidly expanding topics in medicine and human affairs generally (Lilford, 1990). There are many methods for prenatal diagnosis available to study the health status of the fetus. The procedures for prenatal diagnosis are technically complicated because fetuses are protected within the uterus, making a direct examination impossible. This is particularly the case during early pregnancy. In addition, collecting samples of blood, urine or biopsied tissues directly from the fetus as may be required for prenatal diagnosis, requires sophisticated methodology associated with highly skilled practitioners. Furthermore, potential risks exist during the sampling procedure. In the most extreme case, miscarriage may be induced by any faulty management. For the reasons mentioned above, safe and reliable approaches for prenatal diagnosis have to be developed. The following sections describe currently used procedures for prenatal diagnosis (in sections 1.1.1.1 - 1.1.1.5). Chorionic villus sampling (CVS) is the major topic of this study, and will be discussed in detail in section 1.2. It is worthy noting that all the procedures listed below have advantages and limitations; the procedures are therefore often combined together to give a better diagnosis.

1.1.1.1 Ultrasonography

Ultrasonography is a non-invasive method for prenatal diagnosis. Ultrasound waves are directed into the uterine cavity, either using a transabdominal or transcervical probe. The feedback waves are then collected, amplified and displayed onto the attached monitor, providing a transverse or longitudinal "slice" of the fetus and its surrounding environment. This provides a direct, continuous and real-time visualization of the fetus and its associated structures (Richardson, 1991). An ultrasound examination can be safely performed at any stage of gestation. The most popular time for undertaking ultrasonography is between 18 - 20 weeks of pregnancy. This time "window" allows the fetus to develop to a manageable size, and should any structural anomaly be detected, an elective termination of pregnancy can be carried out promptly. It was recently reported that the success rate in the detection of all major morphological anomalies using this approach could be achieved in about 85% of all cases of morphological abnormalities (Chitty *et al.*, 1991; Luck, 1992). In these various studies, the success rate achieved varied according to the characteristic of the individual anomalies being

detected. For example, in cases of anencephaly or renal dysgenesis, the rate of successful detection has usually been relatively high, while in the case of cardiac defects or cases of diaphragmatic hernia, the detection rate was relatively low (Cullen *et al.*, 1992).

1.1.1.2 Maternal biochemical tests

Over the last few years, there has been growing interest in the use of maternal biochemical protein markers for the identification of fetuses in "at risk" pregnancies (reviewed by Canick and Saller, 1993). This method involves collecting a small maternal blood sample and the biochemical detection of "markers" in the serum. For example, open neural tube defects may be diagnosed by the presence of α-fetoprotein (AFP) in the maternal serum and in the amniotic fluid (Wal *et al.*, 1984). The use of gel electrophoresis followed by immunoblotting to detect acetylcholinesterase helps to distinguish open neural tube defects from defects of the anterior abdominal wall (Peat and Brock, 1984). The diagnostic use of AFP has, however, declined recently, due to the efficiency of ultrasonography in the diagnosis of spina bifida and other neural tube defects (Nicolaides *et al.*, 1986a). Nevertheless, cautions have been particularly made when ultrasonography alone is used as a single diagnostic procedure. Thus, current diagnosis often involves a combination of maternal AFP detection and ultrasonographic visualization, the combination approach having a significantly higher detection rate as compared to when each procedure is used alone (Campbell *et al.*, 1987).

In addition to AFP, uE_3 (unconjugated estriol) and hCG (human chorionic gonadotrophin) are also important markers in the maternal blood. A combination of these profiles has been used for the identification of trisomy 21 (Down syndrome), trisomy 18 (Edwards syndrome), trisomy 13 (Patau syndrome), monosomy X (Turner syndrome), triploidy and "open" fetal defects including neural tube defects and ventral abdominal wall defects (reviewed by Canick and Saller, 1993).

1.1.1.3 Amniocentesis

Amniocentesis is routinely performed in the majority of maternity centres, and is the commonest available method for routine prenatal diagnosis. Amniocentesis is normally carried out during the second trimester, i.e. between 15 to 20 weeks of gestation. The procedure of amniocentesis involves insertion of a needle (20 to 22 gauge) into the amniotic cavity under the guidance of a real-time ultrasonography to avoid any possible damage to the placenta or the fetus. Amniotic fluid (usually 15 - 20 ml) is then withdrawn through the syringe attached to the needle. Fetal cells present in the amniotic fluid are isolated and maintained in tissue culture for 1- 4 weeks in order to obtain adequate number of dividing cells suitable for analysis. The analyses include cytogenetic screening for large chromosomal defects such as translocations, inversions, and repetition of large fragments of chromosomes, as well as for the detection of whether there is an increase or reduction of the total

number of chromosomes present. Single gene mutations can also be detected using fluorescence *in situ* hybridization (FISH) or other molecular biological methods. A final result is usually obtainable between about 18 and 22 weeks of gestation, depending on the duration of cell culture.

The most common indication for undertaking amniocentesis is to detect autosomal trisomy or other chromosomal defects. More recently, with the rapid development of novel molecular biological techniques, amniocentesis also provides the opportunity for the detection of genetic rather than cytogenetic disorders. This is particularly the case with the availability of polymerase chain reaction (PCR) technology. Amniocentesis also offers a guidance for the management of red cell allo-immunisation and other immunopathological conditions which may occur in the fetus (Welch *et al.*, 1993). When fetal viral infection occurs, the virus may be cultured from the amnionic fluid and the viral particles can be viewed by direct electron microscopy. Amniocentesis, however, still contributes very little to the diagnosis of open neural tube defects (Crandall and Chua, 1995).

The diagnostic accuracy of amniocentesis has been proven to very high with successful rates of between 99.4% to 99.8% (Rhoads *et al.*, 1989; Canadian College of Medical Genetics and the Society of Obstetricians and Gynaecologists of Canada, 1992). However, a procedure-related risk of miscarriage of around 1% out of all amniocenteses performed during 15 to 20 weeks of pregnancy has been reported (NICHHD, 1979; Tabor *et al.*, 1986).

1.1.4 Early amniocentesis

Early amniocentesis aims to withdraw amniotic fluid prior to 14 weeks of pregnancy, while traditional amniocentesis is recommended to be performed after 15 weeks of gestation. The procedures of early amniocentesis are similar to that employed for undertaking midtrimester amniocentesis but more sophisticated skills in ultrasonography are required (Lilford, 1990).

One major benefit of using early amniocentesis instead of traditional amniocentesis undertaken at a slightly later stage of pregnancy is that it can be performed during much earlier stages of gestation. Therefore, should any defects of the fetus be revealed, an earlier therapeutic termination of pregnancy can be performed so that the damage to the mother, either in mental or in physical aspects, can be minimized. This is due to the fact that the results from traditional amniocentesis can only be obtained during 18 to 22 weeks of gestation, by which time a substantial increase in maternal risk would result following an elective termination of pregnancy if carried out at this time. Furthermore, a pregnant woman can normally feel fetal movements starting from about 16 to 18 weeks of gestation, which may make an induced abortion an unbearable decision for the mother. For these reasons, methods of first trimester prenatal diagnosis, including early amniocentesis, have become more commonly adopted.

Technical difficulties and safety concerns about early amniocentesis, however, have been raised. Higher failure rate of cell culture following sample collection before the 12th week of gestation was observed (MacLachlan *et al.,* 1989). This technical problem, however, may be less of a problem with improvements in cell culture techniques. For example, cells in the amniotic fluid can be concentrated by filtration through membranes before culture in order to minimize the volume of tissue culture medium required and thus facilitate cell culture (Byrne *et al.*, 1991). There are safety concerns that are particularly emphasized and may be difficult to counter. Amniotic fluid leaking following amniocentesis, vaginal "spotting" (with blood) and higher bleeding and miscarriage rates have been major concerns (MacLachlan *et al.,* 1989; Walkinshaw, 1995). Furthermore, animal experiments have revealed that cases of pulmonary hypoplasia were found in the majority of cases when early amniocentesis was undertaken (Hislop *et al.,* 1984). This early concern had not been verified in a subsequent clinical study in which no obvious neonatal lung dysfunction was detected in infants subjected to early amniocentesis (Thompson *et al.* 1991). Recent studies, however, revealed a possible link between early amniocentesis and postnatal pulmonary/respiratory problems. Infants subjected to early amniocentesis require more neonatal intensive care than those exposed to CVS or traditional midtrimester amniocentesis (19 : 8 : 5, repectively; Greenough and Nicolaides, 1997). Higher airway resistance and lower specific conductance were observed in those infants whose mothers received early amniocentesis during their pregnancies (Yüksel *et al.* 1997). It was also reported that more respiratory symptons (1.5 time higher rate) were recorded in those neonates subjected to early amniocentesis compared to those exposed to CVS (Yüksel *et al.,* 1997).

1.1.5 Fetal Blood Sampling

Fetal blood sampling has also been used as a method for collecting fetal cells for prenatal diagnosis. Various names, including cordocentesis, percutaneous umbilical blood sampling (PUBS) and funipuncture, have been used to describe this procedure (Walkinshaw, 1995).

Daffos and colleagues (1983) first demonstrated a safe method for collecting fetal blood by inserting a needle into the umbilical cord. After that, other alternative routes were designed to obtain fetal blood from within the intrahepatic portion of the umbilical vein (Nicolini *et al.*, 1990), or by direct cardiac puncture (Antsaklis *et al.*, 1992). These techniques for fetal blood sampling were performed during the second trimester. They clearly demand highly sophisticated skills and should only be performed in well-established centres where the skills required are readily available (Whittle, 1989). More recently, a pioneer study was undertaken to obtain a fetal blood sample from the embryonic-fetal circulation with the assistance of first trimester transvaginal embryoscopy (Reece, 1997). The embryonic and/or fetal circulation was successfully approached, and in five out of these eight cases (62%), a small blood sample was obtained from each individual.

Fetal blood sampling opens a wide range of possibilities in fetal medicine. The cells obtained from such blood samples can be used for karyotyping, diagnosis for evidence of intrauterine growth retardation, fetal infection and non-immune hydrops fetalis. It is also available for the assessement and therapy in allo-immunisation (Walkinshaw,1995). Procedure-associated fetal loss rate from fetal blood sampling was estimated to be 1-2% (Daffos *et al.* 1985; Maxwell *et al.,* 1991), and the incidence of post-procedure chorioamnionitis was reported to be at about 0.6% (Bernaschek *et al.,* 1995). Pre-term delivery following fetal blood sampling was observered in 9% of cases (Bernaschek *et al.* 1995). It was also noted that severe intrauterine growth retardation occasionally occurred as well as a higher than expected incidence of hydrops and intrauterine death (Maxwell *et al.,* 1991). Chueh and Golbus (1990) estimated that, following fetal blood sampling, the risk of a pre-term labour or a premature delivery due to the stress to the procedure was 12% higher than in non-operated control cases.

1.2 Chorionic villus sampling (CVS)

1.2.1 A brief history of CVS

Chorionic villus sampling (CVS) is a method of obtaining fetal cells for prenatal diagnosis by collecting chorionic villi from the developing placenta during the first trimester of pregnancy. Two approaches, i.e. transcervical and transabdominal CVS, have been developed during the last few decades.

The earliest procedure was undertaken by Alvarez (1964) in an effort to collect chorionic samples for the diagnosis of hydatidiform mole. Alvarez collected chorionic samples from a fetus at about 10 to 12 weeks of gestation, and no complication was reported. After that, Aladjem (1969) obtained chorionic material through the transabdominal approach from 215 third-trimester pregnant women in order to investigate the morphology of the placenta. It was reported that no anomalies of either the fetus or of the placenta were observed (Aladjem, 1969). Independently, a Chinese group developed a transcervical approach to obtain villus tissue for accurately determining fetal sex (Tietung Hospital - Anshan Department of Obstetrics and Gynaecology, 1975; cited in Liu 1991). They inserted a 3-mm rigid metal cannula with blunt opening through the cervix until resistance from the gestational sac was felt, they then applied suction and aspirated small pieces of villus material through a 5-mm syringe attached to the metal cannula. Their successful rate was claimed to be as high as 94%, and their miscarriage rate was stated to be only 6% of all their cases (Tietung Hospital - Anshan Department of Obstetrics and Gynaecology, 1975; cited in Liu, 1991). Subsequent attempts to repeat their procedure by others, however, have been disappointing. In only one third of cases were chorionic villi samples harvested, and perforation of the gestational sac commonly took place, leading inevitably to miscarriage (Liu *et al.,* 1983; Horwell *et al.,* 1983).

The first attempt to obtain chorionic material for prenatal diagnosis of diseases during the first trimester of pregnancy was achieved by Smidt-Jensen and Hahnemann (1984). Two years later, the feasability of undertaking transabdominal chorionic sampling was confirmed by other clinicians (Maxwell *et al.* 1986). Furthermore, it was demonstrated that a rapid karyotype could be obtained using chorionic villi at any gestational stage (Nicolaides *et al.,* 1986b).

In addition to the transabdominal approach for undertaking chorionic sampling, an alternative method, transcervical (i.e. through the cervical canal) was also developed. The natural opening of the cervical canal allows a pathway for obstetricians to reach the uterine cavity with minimal difficulty. With its natural diameter of 2-3 mm, the cervical canal can easily accommodate a range of instruments required for the CVS procedure. Earlier trials to utilize the cervical approach were reported by Hahnemann and Mohr (1969). They designed a hysteroscope of 5-7 mm diameter, with a sucking device and a biopsy knife built within it. All the karyotypes derived from the chorionic villus material obtained using this instrument were reported to be very successful (Hahnemann and Mohr, 1969). Using a similar approach, Kullander and Sandahl (1973) confirmed the feasibility of this approach for obtaining samples for karyotyping from fetuses of between 8 and 20 weeks of gestation. However, the diameter of the instrument was reduced to 5 mm, as an improvement following the original study. While the above authors claimed a high diagnostic success rate, the visualization was very restricted (Kullander and Sandahl, 1973). Similarly, vaginal bleeding was encountered in most of the cases, presumably due to the trauma caused by the instruments. Improved visualization was then achieved by the instillation of a physiological saline solution into the uterine cavity during the endoscopic procedure, and successful karyotyping was demonstrated (Gustavii, 1984). It should not be surprising that, with these large diameter instruments, limited endoscopic visualization and instillation of physiological saline solution into the uterine cavity, the procedure was not only a very cumbersome one, but also risky and time-consuming, and never gained general support.

While the risk remains high, these authors introduced transcervical CVS for the diagnosis of genetic diseases and inborn errors of metabolism. When complemented by ultrasonography, transcervical CVS is currently the most popular approach (Liu, 1991).

1.2.2 Anatomy and histology of the developing human placenta during the period when CVS is performed

Between 9 - 12 weeks of gestation (timed from the last menstrual period), i.e. the period when most CVS procedures are performed, the developing chorionic sac does not completely fill the uterus, and is composed of an outer choronic membrane surrounding the inner amnionic sac and its contents (see Figure 1.1 at the end of this chapter). Between the chorion and amnion is the extraembryonic coelom,

which contains a thick, mucoid-like substance. As the embryo develops, the extraembryonic coelom is readily seen and is located between the thin, wispy, freely mobile amnion and the thicker vascularised chorion.

The chorionic membrane is the structure that surrounds the amnion which, in turn, surrounds the embryo/fetus and contains the amniotic fluid. At earlier stages of pregnancy (5 weeks), there are about 200 chorionic villi equally distributed on the surface of the chorion. As pregnancy advances, the villi differentiate so that those facing the uterine cavity degenerate giving rise to the *chorion laevae*, whereas those located at the proximity of the *decidua basalis* proliferate further and eventually become embedded to form the *chorion frondosum* which will soon form the fetal component of the placenta (Figure 1.1). The *chorion frondosum* contains mitotically active villi and is the preferred biopsy site. Further growth takes place in the *chorion frondosum* due to fresh villus formation from the villus stems, and from the progressive abortization of previously formed villi.

At 6 weeks of pregnancy (dated from last menstrual period, equivalent to 4 weeks after ovulation and fertilization, also termed "post-conception"), the chorionic vesicle begins to intrude into the cavity of the uterus. The decidua covering the vesicle increases in volume and is the *decidua capsularis* and that on the maternal aspect of the vesicle is called the *decidua basalis*, while the *decidua parietalis* lines the remainder of the uterine cavity (Figure 1.1).

At 8 weeks of pregnancy (dated from last menstrual period, equivalent to 6 weeks post-conception), CVS becomes a technical possibility. The *chorion frondosum* is now about 3 - 6 mm in thickness while the *decidua capsularis* is less than 2 mm in thickness.

At 10 weeks of pregnancy (dated from last menstrual period, equivalent to 8 weeks post-conception), the *chorion frondosum* gradually starts to get thinner, and the extraembryonic coleom becomes progressively obliterated.

At 12 weeks of pregnancy (dated from last menstrual period, equivalent to 10 weeks post-conception), the villi of the *chorion frondosum* appear as microscopic stumps, while the extraembryonic coelom disappears between the amnion and the chorion. It is worth noting, however, that the extraembryonic coelom is not completely obliterated until about 16-20 weeks of pregnancy when the *decidua capsularis* fuses with the *decidua parietalis* (Hamilton and Boyd, 1970). The chorionic villus now exhibits a distinctive branched appearance. The surface of the villi is punctuated by small buds consisting of an outer layer of syncytiotrophoblast covering a core of mitotically active cytotrophoblast cells. Within the centre of each villus is a mesenchymal core through which capillaries carrying fetal blood cells course. The mitotically active cytotrophoblast buds provide material suitable for

karyotyping, while the mesenchymal core offers material suitable for both tissue culture and biochemical analysis (Wapner, 1997; England, 1996; Lilford, 1990).

1.2.3 Fetal development during the period when CVS is performed

There are two systems of expressing the gestational stage of a conceptus. One is calculated from date of the last menstrual period, and the other from the time of ovulation and/or fertilization. There are two weeks difference between these two systems. While the majority of clinicians prefer to time pregnancy from the date of the last menstrual period, many embryologists prefer the other system. In this study, the former system is principally used, occasionally accompanied by its equivalent post-conception age.

As mentioned in section 1.2.2, between 9 - 12 weeks of gestation (dated from the last menstrual period), i.e. the period when most CVS are performed, the developing chorionic sac does not completely fill the uterus (Figure 1.1). By definition, at the end of week 10 (dated from last menstrual period, equivalent to 8 weeks of post-conception), the embryonic period is completed and the fetal period begins (England, 1996). The fetal period is characterized by the rapid growth of the fetus (especially between 9-20 weeks of post-conception) and is associated with the further differentiation of those organs and tissues that have formed during the embryonic period.

At the end of week 6 (dated from last menstrual period), the upper limb (forelimb) bud appears, and about 2 days later, the lower limb (hindlimb) bud appears. The core of both structures is derived from somatic mesoderm, and is covered with a layer of surface ectoderm. During week 7 (dated from last menstrual period), the human tail develops. This structure evolves from the caudal part of the body fold or tail fold. The length of the tail is usually at its greatest at this time reaching one tenth of the total body length. The upper limb bud protrudes 90° from the body. An apical ectodermal ridge forms along the peripheral margin of the limb bud and influences the development and growth of the limb. The forearm and hand regions become distinguishable during this week.

During week 8 (dated from last menstrual period), a paddle-shaped hand-plate forms distally in the forelimb. The grooves between the digital rays become deeper, making digital rays recognizable (Figure 1.2). The human limb at weeks 8 - 9 after the last menstrual period (i.e. 6 - 7 weeks post-conception) is developmentally equivalent to an E 12.5 - E 13 (day 13 p.c.) mouse limb (Kaufman, 1992).

During week 9 (dated from last menstrual period), the lower limb forms a paddle-like foot-plate distally, and the grooves between each digital ray become visible (Figure 1.2).

During week 10 (dated from last menstrual period), the grooves between the digital rays of the hand-plate indent proximally, and completely separate the fingers. The length of the head is slightly less than half that of the fetus, which has a CR (crown-rump) length of about 39 mm, and the external genitalia are beginning to differentiate. The tail undergoes regression, due to cell death, and is associated with the development of the buttock region of the embryo.

During week 11 (dated from last menstrual period), the fetus is characterized by the rapid growth of the head which is now seen to be almost half of the total fetal length. As the head extends and the chin is raised from the thorax, the neck develops. The fetus at the 9th week (post-conception) is also characterized by the fusion of the eyelids and the first appearance of fingernails. The grooves between the digital rays of the foot-plate indent further proximally, separating the toes. Distinguishing features of the external genitalia appear during week 9 (post-conception) so that the sex of the individual may be recognized by week 12 post-conception. At the end of week 9, the CR (crown-rump) length of the fetus is about 44- 46 mm.

The week 12 (dated from last menstrual period), fetus has doubled in length compared to that observed at week 9. The fingernails of the upper limb are first evident (the toenails develop slowly, and only appear about 4 weeks later). The midgut, which herniates into the umbilical cord, returns to the enlarged abdominal cavity. The fetus at the 10th week is also characterised by the secretion of bile. The CR length at week 12 is around 60 mm.

During week 14 (dated from last menstrual period), the external ears of the fetus have moved rostrally from the neck onto the side of the head. The eyes have moved from the lateral side of the head towards the front of the face. The CR length at this stage is around 85 mm.

1.2.4 Indications for CVS

Chorionic villus sampling (CVS) is normally indicated for two categories of pregnant women. Firstly, those with an increased risk of chromosomal anomalies are routinely indicated for CVS. This category includes those of advanced maternal age, those with a family history of inheritable diseases, and those that have suffered from fetal chromosomal abnormalities during previous pregnancies. Secondly, fetuses with an increased risk of a specific genetic disease. For example, in the case of cystic fibrosis or muscular dystrophy, both biochemical and DNA tests can be performed using the cells isolated from CVS. It was for this specific purpose that the technique was initially developed in Western countries (Walkinshaw, 1995). Chorionic villi can provide adequate diagnostic material for the detection of cytogenetic errors, genetic mutations and inheritant metabolic disorders (see Kazy *et al.*, 1982).

1.2.5 Procedures for CVS

When CVS was initially developed, it was normally undertaken during early stages of gestation, i.e. weeks 7 to 10 of gestation. Due to subsequent concerns regarding its safety, reported in the literature, the World Health Organization now recommends that CVS should be performed no earlier than week 9 of pregnancy (Kuliev *et al.,* 1996). Recently, most CVS are performed between 9 - 12 weeks of gestation. For example, in the Edinburgh area, CVS is routinely carried out only during the 11th week of pregnancy (personal communication, Dr. W. A. Liston). The sampling "window" is chosen to avoid the high background rate of spontaneous miscarriage which occurs when this procedure is undertaken earlier in pregnancy, but still allows sufficient time for the results to be available within the period that a termination of pregnancy may be performed. Another reason for using this sampling "window" is that, by this stage of pregnancy, the *chorion frondosum* can be easily located by sonography as a hyperechoic homogeneous area. More importantly, CVS undertaken during significantly earlier stages of gestation has been suspected of increasing the risk of inducing craniofacial and limb abnormalities (Wapner, 1997).

1.2.5.1 Collecting samples for CVS

CVS can be performed by either the transcervical or the transabdominal approach (Figure 1.3). Each approach has its pros and cons and in most cases the physician or patient will dictate which approach is used. In approximately 3% - 5 % of patients, clinical circumstances such as the presence of cervical polyps and retroverted uterus with posterior placenta will support one approach or the other (see Table 1.1 at the end of this chapter; Wapner, 1997).

1.2.5.1.1 Transcervical CVS

Transcervical CVS is performed using a thin polyethylene catheter through which a stainless steel stylet is inserted (Figure 1.3). The stylet fits through the catheter and both the polyethylene catheter and the stainless steel stylet have to pass through the cervix. They need to provide sufficient rigidity for adequate passage into the *chorion frondosum* without causing damage to the cervix.

Prior to the performance of CVS, ultrasound scanning is applied to establish the site of the chorion frondosum and to confirm fetal viability. The relative position of the uterus and the cervix is identified, and allows catheter placement parallel to the chorionic membranes. Once the catheter is in place, the stylet is removed and approximately 10 to 20 ml of suction is applied with an attached syringe containing 5 ml of collecting media (Liu, 1991).

1.2.5.1.2 Transabdominal CVS

Transabdominal CVS can be performed using a single needle (single-needle approach) or an outer "guide" needle and an inner smaller sampling needle (double-needle technique). With the single-needle approach, the needle tip is first inserted into the myometrium and then redirected parallel to the membrance with the aid of ultrasound guidance. Once the needle is within the placenta, the stylet is removed and 10 to 20 ml suction is applied with an attached syringe containing 5 ml of medum. The double-needle technique involves an outer needle inserted into the myometrium, while a longer sampling inner needle is passed into the chorion frondosum. The stylet of the sampling needle is then replaced with a syringe and a moderate degree of suction is applied similar to that of the single-needle technique (Liu, 1991).

1.2.5.2 Investigations following sample collection

Most biochemical, cytogenetic, and molecular diagnosis that can be made from amniotic fluid or cultured amniocytes can also be made from chorionic villi. The latter, however, has the advantage of giving earlier results, since sufficient fetal enzyme or DNA is present in villus samples to allow direct sample analyses rather than waiting for later sampling and a long period of tissue culture. The advantage, that CVS can give earlier results, however, cannot be assumed to indicate that the results obtained are any more reliable. Misdiagnoses can occur (Gray *et al.,* 1995). This does not, however, preclude CVS from making prenatal diagnosis, since a result is only validated and used clinically by testing sufficient numbers of samples and by combining other diagnostic approaches, for example morphological examination by ultrasonography.

1.2.5.2.1 Biochemical analyses

Biochemical analyses aim principally to detect defects in protein. Although mutations can produce defects in any kind of protein, it is those mutations that result in a defective enzyme that cause most of the known inborn errors of metabolism. There are two major ways to detect a defective enzyme: (1) to measure enzyme activity directly, (2) to measure the level of some metabolites that accumulate due to an enzyme deficiency. A good example of biochemical analyses is the detection of argininosuccinate lyase deficiency that can be reliably detected by measuring enzyme activity in the fibroblast cells of chorionic villi (Hommes, 1991).

There are close to 50 recognized disorders of metabolism of amino acids and related compounds (Grebner, 1993). Biochemical analyses are available for 23 of these (Grebner, 1993), with the remaining potentially diagnosable in that enzyme activity has been found in normal fibroblasts that are obtainable by culturing chorionic villi.

1.2.5.2.2 Cytogenetic analysis

For cytogenetic procedures, there are two ways to analysize the chorionic villi -- direct preparation without cell culture or preparation following cell culture.

A direct chromosome preparation and analysis can be performed based on the fact that the majority of cells in the chorion frondosum (particularly in Langerhan's layer) are continuously dividing. The samples therefore require no further culture (Simoni *et al.*, 1983; Lilford *et al.,* 1983). The advantage of direct preparation is that the result of chromosomal analysis can be revealed within a very short period, i.e. days. This advantage is particularly evident in that the spontaneously dividing cytotrophoblast buds allow the karyotypes to be completed within 2 to 3 hours. Furthermore, the decidual contamination can be reduced dramatically with culture.

Alternatively, cells obtained from CVS may be cultured overnight and be treated with colchicine to stop chromosomal segregation so that chromosomes can be better prepared for examination during their metaphase.

If an overnight culture does not give sufficient cells for analysis, a longer period of cell culture is needed. This involves segregation of villi by either chemical or physical means, while the mesenchymal cores are desired. A longer period of cell culture, for example 8 days, will generally yield sufficient fibroblasts for chromosomal analyses (Schwab *et al.*, 1984). A longer period of culture also provides opportunities for some modified treatments of chromosomes. The X chromosome, for example, can be better examined when the chorionic villi are co-cultured with methotrexate for the detection of the fragile site in the case of Martin-Bell syndrome (Tommerup *et al.,* 1985). The most important role of longer culture is to distinguish between the chromosome constitution of the cytotrophoblast and the genuine fetal constitution, for example, in cases of restricted placental mosaicism.

Both types of preparations, i.e. with or without cell culture, may, of course, be performed simultaneously, since these two methods examine cells from slightly different sources. This allows observers to compare the results from both studies, providing more cautious validation of results for clinical applications (Wapner, 1997).

During the last decade, the efficiency and accuracy of chromosomal analyses (karyotyping) have been very much improved with the introduction of fluorescent *in situ* hybridization (FISH) and electronmicroscopy, as well as a greater variety of traditional banding techniques. These improvements allow better examination of chromosomal deletions, inversions, and repetition. In many cases, point mutations have become detectable by cytogenetic analyses.

1.2.5.2.3 Molecular diagnosis

In addition to biochemical and cytogenetical analyses, studies using molecular biology techniques have been increasingly commonly employed for more than a decade. Molecular diagnoses have not only extended the potential that cytogenetic analyses cannot give, but also have benefited from their requirement for very little sample material needed for a diagnosis. The followings are most commonly used molecular techniques (Gupta and Bianchi, 1997):

(1) *Southern blotting*: The southern blot technique (named after the investigator Edwin Southern) involves DNA extraction from fetal cells (approximately 20 μg from about 15 or 20 mg of villi sample), which is followed by restriction enzyme digestion (Maxwell *et al.*, 1985). After restriction enzyme digestion, the DNA fragments are separated using gel electrophoresis and blotted onto a nylon filter paper (or a nylon membrane). The DNA fragments can be denatured before or after blotting to generate single-strand DNA molecules so that radiolabelled DNA or RNA probes can hybridize with them. After hybridization, banding patterns are obtained by autoradiography. The banding patterns reflect fragment sizes of DNA sequences that have homology to that particular probe. The presence or absence of specific bands therefore can reflect specific changes within DNA sequences and a mutation can be identified.

(2) *Restriction fragment length polymorphism (RFLP) analysis*: When human DNA is exposed to a restriction enzyme (usually restriction endonuclease), the resultant digested mixture contains many DNA fragments of different sizes. These DNA fragments can be separated by gel electrophoresis and transferred for analysis by southern blotting. Each enzyme cuts DNA only according to the positions of the enzyme's recognition sites. Every human being has his or her own unique pattern of cleaved DNA fragments that can be traced from the patterns of his or her parents. If a mutation occurs, the normal recognition sites for a specific enzyme can be alterred by the change of DNA sequences. Thus, the RFLP pattern will be changed and become different from to the patterns of his or her parents. Therefore, a genetic defect can be identified.

(3) *Polymerase chain reaction (PCR):* For smaller amount of villi cells, PCR is available to provide genetic analyses (reviewed by Ehrich *et al.*, 1991). PCR involves *in vitro* amplification of minute amounts of DNA to generate sufficient quantities of signal to permit detection by more traditional methods. The technique uses relatively heat-stable enzymes from bacteria, for example Taq polymerase, to amplify DNA defects to a detectable level by repetitively copying template strands of DNA. Following amplification (usually more than a million duplications of the target sequence within hours), the sample DNA can be processed through Southern blotting, DNA or RNA probing and RFLP analyses. Because the initial amount of DNA is so small, PCR is particularly useful in

CVS. Firstly, the quantity of clinical material can be extremely small. Secondly, PCR can considerably reduce the time required from sample acquisition to results because large amounts of DNA can be obtained without culturing cells.

1.2.6 Safety concerns for CVS

1.2.6.1 Pregnancy loss following CVS

The side effect of most concern related to the procedures of CVS is the suspected higher rate of pregnancy loss. The concern, however, has been alleviated due to several large demonstrations of data evaluating the relative safety of CVS when compared to amniocentesis (Wapner, 1997).

CVS is carried out during a time "window" within which a variety of spontaneous miscarriages could occur, and there is no doubt that the earlier the gestation is when CVS is undertaken, the higher this background pregnancy loss rate would be (Stranc *et al.*, 1997). Thus, to distinguish CVS-induced miscarriages from background spontaneous pregnancy loss is necessary.

Low pregnancy loss rates following CVS have been reported by individual centres (Clark *et al.*, 1989; Green *et al.*, 1988; Gustavii *et al.*, 1989; Jahoda *et al.*, 1989; Young *et al.*, 1991). In general, reports from experienced workers documented pregnancy loss rates following CVS within the range between 2 % and 3 % before 28 weeks of gestation (Jackson *et al.*, 1992).

A number of surveys from multiple centres were conducted to compare the safety and accuracy between amniocentesis and CVS (Canadian Trial, 1989; Rhoads *et al.*, 1989; MRC Working Party, 1991; Lippman *et al.*, 1992). Diagnostic accuracy was slightly higher in amniocentesis (99.4-99.8%) compared with that seen in the CVS (97.5-97.6%). The reasons for this may be due to higher frequency of repeated procedure, mosaicism and contanimation by maternal cells. Nevertheless, it is inevitably difficult to compare the impacts of these two procedures on the developmental fate of embryos since CVS and amniocentesis are performed at different stages of pregnancy. In order to ascertain the incidence of baseline pregnancy loss during these two separate periods, all patients should be included in trial studies well before the gestational age at which CVS is normally performed, and each individual pregnancy loss caused by any reason should be fully recorded (Wapner, 1997). This would allow researchers to eliminate bias between the two procedures when these take place at significantly different pregnancy stages. For example, a spontaneous miscarriage or an elective termination of pregnancy following the detection of abnormal results could be excluded, as well as embryos with an abnormal karyotype spontaneously aborted prior to an amniocentesis (Waper, 1997). In North american trials, in desired pregnancies, a slightly excess fetal loss rate was reported in CVS cases, i.e. between 0.6% (Canadian Trial, 1989; Lippman *et al.*, 1992) to 0.8 %

(Rhoads *et al.*, 1989) above that of amniocentesis cases. On the evaluation of CVS by the European studies, a greater 4.6% of total pregnancy loss was observed in CVS compared with that of amniocentesis (MRC Working Party, 1991). This was divided into an increased incidence of spontaneous deaths before 28 weeks of gestation (2.9%), more elective terminations of pregnancy due to abnormal choromosome constitution (1.0%), and more neonatal deaths (0.3%) following CVS procedure. These discrepant findings might be explained by the smaller number of cases being performed in each centre in the European Working Party study; half of the size compared with the Canadian Trial and less than one sixth compared with the American study. In addition, as no obvious increase in pregnancy loss rate was noted during the period of the European trial, it has been suggested that a "learning curve" should be required for both the transabdominal and transcervical CVS with at least 400 cases being in this category (Saura *et al.*, 1994; Wapner *et al.*, 1994).

In other prospective comparative studies, in which both CVS and early amniocentsis (details see below) were carried out at similar gestational stages between 10 and 13 weeks of pregnancy, the comparison between these procedures showed that the fetal loss was higher (5.3% vs 2.3%, respectively; Nicolaides *et al.*, 1994) and repeat testing was needed more frequently after early amniocentesis (Cederholm and Axelsson, 1997). Recently, numerous studies were also undertaken to evaluate whether the pregnancy loss rate was different between transabdominal and transcervical CVS, but no significantly different findings were reported (Brambati *et al.*, 1990; Brambati *et al.*, 1991; Jackson *et al.*, 1992; Smidt-Jensen *et al.*, 1994; Wapner *et al.*, 1994). An increased risk of the transcervical procedure over transabdominal one was demonstrated in two prospective, randomized, large-scaled studies (Church *et al.*, 1995; Smidt-Jensen, 1991). It had been suspected that this finding was due to limited experience in the transcervical procedure in those establishments, and therefore it had been speculated that fetal survival rate could be improved after the operators had obtained more extensive experience; the conclusion was drawn that these two techniques to serve as 'the most complete, practical, and safe approach to first-trimester diagnosis' (Wapner, 1997).

1.2.6.2 Fetal abnormalities following CVS

Within 289 babies who received CVS between 56 to 66 days of gestation, Firth and co-workers (1991a) reported that 5 of them were born with craniofacial and limb defects. Four cases exhibited features similar to those observed in the oromandibular-limb hypogenesis syndrome (OMLHS; also termed oromandibulo-facial limb hypogenesis -- OMFL --syndrome), and the other presented a terminal transverse limb reduction defect. All infants possessed a normal chromosome constitution. When the incidence of craniofacial and limb defects following CVS in their study (1.7%) was compared to that of the general population (see below), it was found to be significantly higher, and this therefore raised the possibility that the CVS procedure might produce abnormalities similar to those observed in the OMLHS. In the original study in which the spontaneous occurrence of OMLHS

syndrome was surveyed, the frequency of this syndrome was estimated to be about 1 in 175,000 livebirths (Froster-Iskenius and Baird, 1989; Froster & Baird 1992), and limb reduction defects to be at 1 in 1690 livebirths (Hoyme *et al.*, 1982). It also appeared in the literature that a considerable number of reports supported this hypothesis (Planteydt *et al.*, 1986; Christiaens *et al.*, 1989; Kaplan *et al.*, 1990; Mahoney, 1991; Editorial, 1991; Hsieh *et al.*, 1991, 1995; Mastroiacovo and Cavalcanti, 1991; Rodriguez and Palacios, 1991; Brambati *et al.*, 1992; Burton *et al.*, 1992, 1993; Kuliev *et al.*, 1992; Mastroiacovo *et al.*, 1992; Gruber and Burton, 1994; Firth *et al.*, 1994; Mastroiacovo and Botto, 1994; Olney *et al.*, 1995; Botto *et al.*, 1996). In a case-control study, using the information available from Italian multicentre birth defects registry, an odd ratio of 11.3% (95% CI 5.6 to 21.3) for transverse limb abnormalites has been shown in post-CVS cases (Mastroiacovo *et al.*, 1992).

The relationship between CVS and the induction of limb abnormalities in particular remains controversial for several reasons. Firstly, there are reports which appear to indicate no significant increased risk of OMLHS abnormalities following CVS (Rhoads *et al.*, 1989; Schloo *et al.*, 1992; Holmes, 1993; Jackson *et al.*, 1993; Report of NICHHD Workshop, 1993; Froster and Jackson, 1996), and secondly, the "baseline level" (i.e. procedure-related vs spontaneous incidence) of similar abnormalities has been criticised because different populations were analysed, and different classification criteria were employed in the various studies; for example, Firth and colleagues drew their conclusions using the baseline of incidence in the general population provided in the other study reported by Froster-Iskenius & Baird (1989) (see Froster and Baird, 1992; Hsieh *et al.*, 1995). Recently, Hsieh *et al.* (1995) published findings in which matched control groups were studied, and found that the incidence of limb defects after CVS was significantly higher. This was particularly evident in relation to the most severe types of lesions, namely amelia and transverse limb reduction defects, which had been induced when CVS exposure had occurred no later than week 9 of gestation, with an 88-fold increased incidence in the CVS-exposed compared to a matched control groups. Botto *et al.* (1996) have recently argued that the various negative reports available in the literature only considered the most severe types of limb defects, such as transverse limb reduction deformities, and accordingly considerably *underestimated* the risk of induction of all types of limb deformities following CVS. In Botto *et al.*'s (1996) study in which limb defects of all degrees of severity were included where two different definitions of transverse digital deficiencies, i.e. (i) one or more digits (any TDD) and (ii) all five digits in at least one limb involved (extensive TDDs), these authors reported that a high relative risk existed whichever criteria was employed. They thus suggested that the classification criteria should be re-evaluated and the associated risk between CVS and limb abnormalities definitively determined. Thirdly, the gestational age of sampling may play an essential role in ascertaining the relationship between CVS and limb defects. When the data was analysed according to the gestational age at CVS, a considerably increased risk of transverse limb reduction defects (19.7%) was observed in the infant who received CVS procedure prior to day 70 of pregnancy, while in those who had a delayed CVS procedure (i.e. after day 70 of pregnancy), no increased rick

was found with regard to this aspect (Dolk *et al.*, 1992). Similar observations were also encountered in an exceptionally experienced establishment. Brambati and colleagues (1992) reported that no increased risk of limb defects in association with infants whose chorionic villi samples were collected after 9 weeks' of gestation, while in the goup where samples were harvested at week 6 and 7 of pregnancy, a 1.6% incidence of limb defects was found. In addition, in the Taiwanese report (Hsieh, *et al.*, 1995) all infants displaying OMLHS syndrome were exposed to CVS certainly before day 63 of gestation, although an accurate date for each case could not be traced. Finally, the experience of the operators may contribute a bias on the cooperative studies between centres. Wapner (1997) reviewed the 29 infants reported by the Taiwanese group, and emphasised that all these cases were carried out in the community-based centre, while no abnormalities were encountered in the major medical teams. He then suspected that excessive placental trauma caused by less experienced operators may have played a critical part in the reported clusters of post-CVS limb reduction defects.

1.2.7 Pathogenesis of CVS-induced abnormalities

1.2.7.1 Bleeding

Post-CVS bleeding may present in different ways, with or without being noticed by the patient herself. A subchorionic haematoma had appeared immediately after transcervical CVS in about 4% of patients (Brambati *et al.*, 1987). This normally disappears before the 16 weeks' of gestation, without any adverse outcome (Brambati *et al.*, 1987). Minimal "spotted" bleeding may account for the most common occurrence, and have been repoted in almost one third of pregnancies following transcervical CVS (MRC Working Party, 1991; Rhoads *et al.*, 1989). A small amount of vaginal bleeding was reported in 2 % of cases subjected to the transabdominal approach while in as many as in 7% of women after the transcervical method (MRC Working Party, 1991; Rhoads *et al.*, 1989).

In most of the cases indicated above, CVS-related bleeding stops within a limited period, and probably contributes no harm to the pregnancy outcome. In the experience of 14,000 cases undertaken at a well-established centre, no elective termination of pregnancy, nor re-admission of patients due to post-procedure complications has ever been required (Wapner, 1997). Severe bleeding was observed in the patients when the practitioner misplaced the transcervical catheter into the vascular decidua basalis attached to the chorion frondosum. The warning sign of a 'gritty' feeling during the introduction of the catheter into the sampling site is normally an indication of the latter accidental situation. It has been advised that the transcervical catheter should be introduced with great care to avoid unnecessary contact with other structures, and to minimize the risk of hemorrhagic complications (Wapner, 1997).

1.2.7.2 Infection

The vaginal passage is a natural incubator of microorganisms, and concerns have been raised that vaginal flora could possibily be introduced into the uterine cavity during the transcervical CVS procedures. This possibility has been confirmed by the findings that bacteria were isolated in 30% of transcervical catheters (Brambati *et al.,* 1990; Brambati and Varotti, 1985; Garden *et al.,* 1985; Scialli *et al.,* 1985; Wass and Bennett, 1985). Fortunately, the contamination of the instrument has a very weak connection in regard to the clinical outcome, i. e. the incidence of chorioamnionitis following CVS was low (Brambati and Varotti, 1985; Canadian Trial, 1989; Hogge *et al.,* 1986; Rhoads *et al.,* 1989). The infection was suspected to correlate with only 0.3% of pregnancy loss in a study of over 2000 cases of transcervical CVS (Rhoads *et al.,* 1989).

Predictably, when compared with transcervical approach, the transabdominal method provides more aseptic potential than the latter, since the abdominal wall can be thoroughly cleaned before the procedure. A very few cases of infection after transabdorminal CVS were reported. The etiology was believed to be a contamination of bowel flora introduced by the sampling needles secondary to an inadvertant puncture of the bowel. In fact, the incidence of post-CVS chorionamnionitis remains at a very low level, and therefore no routine screening for any organism apart from gonococcus is indicated (Wapner, 1997). Two life-threatening cases of septic shock were reported following transcervical chorionic biopsy at its initial developmental stage. Similar clinical pictures to the chorioamnionitis seen were observed in cases with retention of intrauterine contraception device (IUD). In addition, repeated insertion of a single transcervical catheter had taken place in these two patients (Barela *et al.*, 1986; Blakemore *et al.*, 1985). To learn lessons from these two cases, the practice that a new sterile catheter should be applied in each single insertion has been widely adopted, and thus only extremely rare cases of CVS-related pelvic infection were encountered. For example, no severe infection following CVS was observed in 4268 cases in a multicenter collaborative study (Rhoads *et al.*, 1989).

1.2.7.3 Rupture of membranes

Although the results from intentional puncture of chorionic and/or amniotic membrane using transcervical catheter revealed that these membranes possess great resistance to the brutal manoeuvres without the formation of perforation, several cases of acute rupture of the membranes following CVS were observed either in the form of obvious leakage of amniotic fluid or decreased volume of amniotic fluid detected by ultrasound late in pregnancy (MRC Working Party, 1991; Rhoads *et al.,* 1989). It has also been recognized that delayed rupture of the membranes often occurs days or weeks after CVS and thus was considered as one possible complication of the CVS procedure.

The incidence of delayed rupture of the membranes after CVS has been reported to be between 0.3 % (Brambati *et al.,* 1987b; Hogge *et al.,* 1986; MRC Working Party, 1991) and 0.7 % (Rhoads *et al.,*

1989) by individual groups and collaborative surveys. A further unexplained delayed midtrimester oligohydramnios has been observed as an uncommon complication of CVS (Cheng *et al.*, 1991). It was believed that the midtrimester oligohydramnios was due to a delayed chorionamnion rupture described above and a succeeding slow leakage of amniotic fluid (Cheng *et al.*, 1991).

1.3 OMLHS (oromandibular-limb hypogenesis syndrome) and its association with CVS

1.3.1 A brief description of the OMLHS syndrome

OMLHS is one of the subtypes of hypoglossia-hypodactyly syndromes (also termed oromandibulo-facial limb hypogenesis -- OMLF -- syndrome, Winter and Baraitser, 1991). It is easily confused with another congenital syndrome called aglossia-adactylia. In the case of OMLHS, the tongue is seldomly absent, although its size is small.

The clinical findings of OMLHS present a spectrum of changeable features. This is particularly true in the limb defects that can range from deficiency of digits to the transverse limb reduction defects involving the distal parts. The lower jaw is inevitably small. However, no intellectual impairment was observed in those infants who survived. A certain linkage exists between OMLHS and the Moebius syndrome, in which bilateral facial and abducens cranial nerve palsies are normally found.

The general features of OMLHS are as the followings:

Absent fingers or oligodactyly
Absent nails
Absent or hypoplastic tongue
Bound tongue
Cleft palate
Facial weakness
Hypodactyly (the presence of the less than the normal number of fingers or toes)
Microstomia (a congenital defect in which the mouth is unusually small)
Oligodontia (absence of many teeth, usually associated with small size of the existing teeth, and other anomalies)
Oral frenulae
Oral synechia (i.e. fusion of parts)
Reduction deformity of arms, no digits
Reduction deformity of legs, no digits
Skin syndactyly of fingers

Small mandible/micrognathia

The spontaneous occurrence of the OMLHS syndromes has been reported as sporadic (Winter and Baraitser, 1991), with an incidence in the general population believed to be 1 in 175.000 live births (Froster-Iskenius and Baird, 1989; Froster and Baird, 1992).

1.3.2 Clinical findings of CVS-induced abnormalities similar to OMLHS syndrome

Systematic anomalies were observed clinically in the infants who displayed OMLHS syndrome after their exposure to CVS procedures. The most typical abnormalities observed in the head region were different degrees of cleft palate, cleft lip, microglossia and micrognathia (Firth *et al.,* 1991b). Brain stem infarction was also occasionally noted, while in the trunk region, the most common feature was abdominal wall defect. In the extremities, a series of characteristic limb defects has been reported (Planteydt *et al.,* 1986; Christiaens *et al.,* 1989; Kaplan *et al.,* 1990; Mahoney, 1991; Editorial, 1991; Hsieh *et al.,* 1991, 1995; Mastroiacovo and Cavalcanti, 1991; Rodriguez and Palacios, 1991; Brambati *et al.,* 1992; Burton *et al.,* 1992, 1993; Kuliev *et al.,* 1992; Mastroiacovo *et al.,* 1992; Gruber and Burton, 1994; Firth *et al.,* 1994; Mastroiacovo and Botto, 1994; Olney *et al.,* 1995; Botto *et al.,* 1996). For examples, in four fetuses described by Firth and her collegues (1991b), various type of limb defects were found. Some exhibited transverse limb termination with vestiges of all 5 digits; some presented with hypoplastic digits, with syndactyly involving the distal inter-phalangeal joints, and others showed disorganised foot below the ankle region without any recognisable digits (Firth *et al.,* 1991b).

Clearly, in the less severely affected infants, only a selection of the abnormalities might be expected to be observed. The types of limb abnormalities observed in the affected infants appeared to be related to the time during pregnancy when CVS was carried out (summarised by Firth *et al.,* 1994; Hsieh *et al.,* 1995; Botto *et al.,* 1996; also see below). The earlier this procedure was undertaken, the more proximal the lesions encountered tended to be. Thus, transverse limb reduction defects were invariably seen in those infants who had been subjected to CVS before or during week 9 of gestation, whereas relatively minor limb defects (such as finger nail hypoplasia) was seen when exposture to CVS procedures occurred slightly later in gestation. A full range of limb defects was observed in all these clinical studies following CVS. The limb defects ranged from the most severe cases, for example transverse limb reduction defects, through soft tissue syndactyly, brachysyndactyly, and adactyly to nail hypoplasia in the least affected infants.

The incidence of OMLHS observed following CVS varied (see section 1.2.6.2), ranging between 0.09 % (Schloo *et al.,* 1992) to 1.7 % (Firth *et al.,* 1991a). Although some contradictory view in terms of

the association of CVS with OMLHS have been raised by some authors as described in section 1.2.6.2, it is beyond doubt that the risk of inducing OMLHS in the CVS-exposed babies exists, although the incidence is low and varied between the different reports.

1.3.3 Possible explanations for the CVS-induced abnormalities

1.3.3.1 A postural basis for the craniofacial deformities

Several hypotheses have been advanced to explain the link between CVS and the OMLHS syndrome. In early animal studies, cleft palate was induced in the rat at a rate of 100 % by the amniotic sac puncture when this was carried out immediately prior to palate closure (Poswillo, 1966). Detailed evidence of compression by the amnion and/or the uterine muscles on the embryo has been given both on the macroscopic and the microscopic level. Macroscopically, when the mandible was dissected away from the skull, the tongue was found wedged between the two palatal shelves. After the tongue was lifted, a wide gap between the two posterior palatal shelves was clearly shown. Both the shape and the size of the cleft palate matched accurately that of the intervening tongue, suggesting that the mechanism of cleft palate was due to the compresion of the amnion and/or the uterine muscles. Other evidence of compression was also observed by Poswillo (1966). The premaxillary shelf was compressed, though intact. The skin on the face, head and body showed evidence of folding and wrinkling.

Microscopically, Poswillo (1966) described three lines of evidences to support the hypothesis that cleft palate was caused by mechnical tension by the constriction of the extraembryonic membranes and/or the uterine musculature. Firstly, the tongue was found to be wedged between its surrounding structures, including two lateral palate shelves, the above nasal septum and the floor of the mouth below. Secondly, the shape of the nasal septum, which was usually straight in the normal embryos, twisted in the compressed embryos and formed abnormal nasal cavities. Thirdly, the shape of the thoracic wall exactly mirrored the distorted pattern of the lower boundary of the mandible.

Similar observations were also found in previous studies, in which amniotic sac puncture was performed with or without withdrawal of amniotic fluid (Walker, 1959). Punctured embryonic units were either released from the uterine horn and exposed in the maternal abdominal cavity, or remained within the uterine horn to allow further development. Cleft palate was observed in both groups. The results were interpreted by Walker (1959) as being due to the fact that the compression of the amnion alone (or together with that of the uterine muscle) forced the head towards the chest, driving the tongue between the palate shelves and subsequently leading to cleft palate. In addition, Walker (1959; 1967; 1968) proposed that in order to achieve normal palate closure, extension of the neck was

essential. This could elevate the head away from the chest and thus allow space for the movements of the tongue and lower jaw to occur (Walker, 1967; 1968).

To test this hypothesis, experiments were set up to investigate the correlation of the mouse embryonic movements with palate closure following the administration of various anaesthetic procedures. Embryos with reduced reflexogenic movements of the neck region resulted in cleft palate and cleft lip (Walker, 1969), supporting the previous hypothesis.

1.3.3.2 A vascular etiology for the limb abnormalities

While the cleft palate, cleft lip and abdominal wall defects might have been had a postural basis, many authors favour the vascular aetiology as a direct or indirect cause of limb abnormalities (Firth *et al.,* 1991b; Burton *et al.,* 1992; Brent 1993; Report of NICHHD Workshop, 1993; Kaufman, 1994).

One possibility exists, that the damage to the site of sampling during the CVS procedure could stimulate the release of vasoactive factors, for example prostaglandins, into the fetal circulation. Alternatively, fetal blood loss, or thrombosis within the vessels close to the site of sampling might induce either fetal hypotension, or embolisation, hypoperfusion and finally leads to anoxia of the extremities (Firth *et al.,* 1991b).

Long before the CVS technique became widely available, Hoyme *et al.* (1982) reported that four fetuses born with unilateral transverse limb reduction defects were strongly suspected to be associated with thrombus formation within the placental bed. One of them displayed abnormal chromosomal constitution and aborted at 80 days of gestation. Multiple areas of cutaneous petechiae (a pinpoint, non-raised, perfectly round, purplish red spot caused by intradermal or submucous hemorrhage) were observed through the whole body of the aborted fetus. The other three were delivered without complications and no other morphological anomalies were observed. Radiographic analyses of their forearms revealed that the structure of the humerus was absolutely normal and the size and configuration of the radius and ulna was also normal in the remaining stumps. Complete function of elbow joint including flexion, extension, supination and pronation were presented. Although intact membrane of chorion and amnion and grossly normal placenta were present, microscopic examination of the placenta disclosed striking features. Multiple centres of organized thrombi were observed over most of the large fetal mainstem villous vessels, and placental infection such as chronic villitis or basal deciduitis was also confirmed. A bidirectional Doppler technique was used to study the blood flow of the stumps, and the results showed that normal blood flowed down to the bifurcation of the brachial artery, but no further blood flow could be detected beyond that level in either the radial or ulnar arteries. All these findings, though the mechanism remained unknown, strongly suggested that a previously normally formed structure (distal part of limb) was interrupted by the intrauterine

vascular disruption with a subsequent occlusion in the brachial artery. The disruption and occlusion results in ischaemia and necrosis in the distal limb areas, and finally a resorption of the structures distal to the site of thrombosis.

In the rat, Brent and Franklin (1960) first described the teratogenic effects of clamping of the uterine vessels for a period between 1/2 to 3 hours. Subsequent study was carried out by Leist and Grauwiler (1974) during days 14 - 16 of pregnancy in the rat. Their results revealed that clamping the uterine vessels for 45 minutes produced a series of haemorrhagic lesions and tissue necrosis on the fetal extremities including nose, limbs, tail and genital tubercle. And occasionally in the most severe cases, the clamping procedure resulted in the reduction amputation, or fusion of the distal parts of the limbs.

Similar findings were observed in a further detailed experiment, in which various anesthetics and diffierent degrees of manipulation on the uterine blood vessels were applied (Webster *et al.*, 1987). Although varied anesthetic reagents (sodium pentobarbitone and a ketamine/rompun/atropine mixture) and a full range of clamping methods with gradual severity were used, haemorrages were clearly demonstrated in the foot plate of the hindlimb in most of the fetuses, as well as on the face, forelimbs, tail and genital tubercle in the severely affected cases. When the fetuses were allowed to develop near term, the affected offspring exhibited amputation, hypoplasia and syndactyly of digits, which were similar abnormalities to those seen in the fetuses previously subjected to amniotic sac puncture (references see above).

Furthermore, similar observations were also found in the fetuses exposed to interruption of the normal uterine blood flow by vasoconstriction (adrenaline or vasopressin). Therefore it has been suggested that the vascular disruption of the uterine blood circulation may contribute a temporary hypoxia, bradycardia, and serum ion changes in the fetuses, leading to hypoplasia, amputation, necrosis and resorption (Leist and Grauwiler, 1974).

The only *direct* evidence provided so far with respect to the relationship between haemorrhagic lesions and placental trauma in human limbs was observed using embryoscopy (Quintero *et al.*, 1992). In their preliminary experience, fetal ecchymotic lesions (i.e. ecchymosis: a small hemorrhagic spot, larger than a petechia, in the skin or mucous membrane forming a nonelevated, rounded or irregular, blue or purplish patch) located on the calvarium, face (philtrum), thorax and the distal segments of the limbs were observed after transcervical CVS carried out just before termination of pregnancy at 9.5 weeks of gestation. Embryoscopic observation indicated that the post-CVS bruises on the fetus became larger during the 10 minute period of inspection, and the development of a subchorionic haematoma during CVS was fully recorded under ultrasonography.

To test whether the damage of the placenta during CVS would produce fetal haemorrhagic lesions, the authors deliberately created partial placental detachment and subchorionic haematoma with a blunt instrument during CVS on seven conceptuses shortly before their elective abortion carried out between 8 to 12 weeks of gestation. While no haemorrhagic lesions were noted on these cases before CVS, six of them displayed ecchymoses on the head, face and thorax (but not on the limb) region. In one of the latter, additional haemorrhagic injuries were also observed extending along the tract of vitelline vessels and on the yolk sac. This study demonstrated a direct link between placental trauma in early pregnancy and the resultant ecchymotic lesions on the fetus, though its clinical significance and underlying mechanism remained unclear. It has been suggested by the authors that, although some of the ecchymotic lesions may not have been significant, others may well form the pathological basis for limb reduction anomalies and oromandibular hypogenesis syndrome. Other factors such as gestational age, operator's skill, the degree of placental trauma, and/or the appearance and location of subchorionic haemorrhage, however, were also nominated by these authors to justify any possible relationship observed.

1.3.3.3 Other possibile cause of the limb abnormalities seen following CVS

Others have suggested that the anatomical feature of the exocoelomic gel which is located between the chorion and amnion becomes obliterated by about day 56-63 of gestation (Shepard *et al.*, 1991; for anatomical details see section 1.2.2). It was hypothesized by these authors that the obliterated exocoelomic gel may entrap one or more of the distal extremities and subsequently result in tissue ischaemia and necrosis (Shepard *et al.*, 1991; Burton *et al.*, 1992). Although this suggestion, may explain the findings presenting in the limbs, it cannot account for the craniofacial abnormalities encountered in those clinical studies.

Recently, a hypothesis has been proposed based on the finding that in 20% of CVS-exposed infants, their circulation contains a dramatic increase in the level of maternal cells (Los *et al.*, 1996). This implied that maternal serum may act with the fetal blood group antigens immunologically through the materno-fetal transfusion induced by CVS. An animal model was designed to test this hypothesis. An intracardiac injection of maternal antisera was administered into the 10-somite stage rat embryo which were retained in a whole-embryo culture system (van der Zee *et al.*, 1997). At this stage of development, the dorsal aorta is believed to possess an intact endothelial lining, while the pharyngeal arch arteries and the limb vessels still possess a fenestrated epithelium. Based on this feature, the authors proposed that this might involve the vascular interruption at the craniofacial and limb sites following cvs. It is unclear whether the immunological mechanisms proposed in this hypothesis are comparable with the earlier stage in the rat model system and that in the human fetus at the time when CVS is usually performed clinically.

1.3.3.4 The most plausible hypothesis for the limb abnormalities

The most plausible hypothesis that has been proposed to date is that an accidental rupture of amnion takes place when CVS is performed (Shepard *et al.*, 1991), leading to oligohydramnios and compression of the embryo by the extraembryonic membranes and uterine muscle, resulting in hypoperfusion and hypoxia of tissues (Kaufman, 1994). It is also suggested that the latter might lead to immobilisation and postural remodelling of the embryos. This possibility may not only explain the limb defects, but also explain the craniofacial abnormalities reported in the clinical studies.

1.3.3.4.1 The reasons why the latter hypothesis is preferred

Based on the anatomical structure of the chorion at the stage when CVS is normally carried out clinically (starting from week 8), it is quite likely that the surgeon might rupture the amnion without noticing it. As indicated above (see section 1.2.2), it was estimated that at 8 weeks of gestation, the thickness of the chorionic frondosum is 3-6 mm while the decidua capsularis measures less than 2mm (Hamilton and Boyd, 1970). At 10 weeks of pregnancy, when the CVS procedure is probably most optimally undertaken, the chorion laevae has become much thinner. By the end of the first trimester of pregnancy, the villi have diminished to microscopical stumps, and the extra-embryonic coelom has disappeared due to the fusion of the chorion and amnion.

Furthermore, should any inadvertent rupture of the amnion occur during CVS, neither the clinician nor the patient would necessarily be aware that this has occurred. At the end of week 8 of pregnancy, the volume of amniotic fluid has been estimated to be around 10 ml (range from 4 - 40ml; Chamberlain *et al.*, 1984; Brace and Wolf, 1989; Gilbert and Brace, 1993), which is a relatively small amount to be noticed if the amnion has been inadvertently damaged. This suspicion has been confirmed by the findings of amniotic membrane rupture following cvs (details see 1.2.7.3). The incidence of amniotic fluid leakage after the CVS procedure has been reported in two clinical studies, ranging from 0.3 % (MRC Working party 1991) to 0.7 % (Rhoads *et al.*, 1989). Besides, it is not uncommon that the leakage of amniotic fluid, should it arise, could be easily disguised by the vaginal bleeding following CVS. The MRC Working Party (1991) reported that 2 % of patients bled after transabdominal CVS and 7 % following transcervical cvs.

Additional supporting information, though indirectly, could also be obtained from one of the explanations regarding the pattern of abortion observed following invasive prenatal tests including the CVS procedure. One kind of the abortion happens within the first week after CVS, and is usually accompanied by vaginal bleeding followed by abortion. Equally common, the other type of abortion is normally noticed between 1-5 weeks after the procedure, with the fetus growing initially, but severely affected by the sequences of oligohydramnios, loss of fetal heart rate and finally abortion. The latter

type has been suspected as a consequence of the chronic infection by organisms which are existing in the vaginal areas, such as Listeria, Chlamydia or Mycoplasma (Scialli *et al.*, 1985).

1.3.3.4.2 Evidence from the animal models

Supporting data have accumulated in a series of animal studies to examine the effects of amniotic sac puncture with or without active withdrawal of amniotic fluid. These studies have been carried out at a corresponding stage when the CVS would normally be performed clinically (mouse: Trasler *et al.*, 1956; Walker, 1959; MacIntyre *et al.*, 1995; rat: Poswillo and Roy, 1965; Poswillo, 1966, 1968; Kendrick and Feild, 1967; DeMyer and Baird, 1969; Love and Vickers, 1972; Singh and Singh, 1973; Singh *et al.*, 1974; Kino, 1975; Kennedy and Persaud, 1977; Houben, 1980, 1984; Houben and Huygens, 1987).

In the latter studies, comparable craniofacial and limb defects have been reported, and the results demonstrated that the most plausible mechanism underlying these findings was based on the vascular disuption. Examination of external morphology of the fetuses subjected to the experimental procedure demonstrated the presence of cutaneous bruising ranging from mild petechiae to severe ecchymoses were observed. This was present on the cephalic and trunk areas, and as well as on the whole limb and its very distal extremeties. These haemorrhagic lesions appeared initiated shortly (15 minutes) after ASP and often lasted for a further period of 48 hours.

Histological analysis carried out on these fetuses, with particular interests on the limbs, revealed that a variety of vascular disruptions occurred immediately following the amniotic sac puncture. Within 2-3 minutes after the experimental procedure, vascular endothelial tears were observed in the marginal vein surrunding whole autopod, the interdigital capillary network, and also between the apical ectoderm ridge and mesenchymal tissues subjacent to it. Larger areas of haemorrhages appeared in the interdigital zones, and became increasingly obvious at later intervals following the experimental insults.

Skeletal analysis on the affected limbs of human fetuses or in the experimental animals at later gestational stages that had been subjected to amniotic sac puncture previously revealed unexpected findings (Kino, 1975; Love and Vickers, 1972; Houben, 1984). In a comparative study between human and rat limbs, using radiography, Kino (1975) demonstrated that the syndactyly/adactyly findings in the affected cases, was exclusively related to the soft tissues in the interdigital zones. The cartilaginous and bony elements were not involved in the traumatic procedures. However, the author reported the observation of a skeletal "bridging" element intevening between two digits (termed acro-syndactyly) in some of the human and rat cases. In addition, in the advanced rat fetuses, a skeletal dichotomy (splitting of usually the distal phylangeal elements) were noted in some of the digits.

1.4 Aims of this study

It remains unclear why the removal of a small amount of chorionic villus sample could produce infants with congenital abnormalities. It is also interesting to notice that in the experimental literature, a single puncture of the amniotic sac with or without withdrawal of fluid could induce such a wide range of abnormalities involving various parts of fetuses or newborn animals. Based on the fact that similar features have been produced in a proportion of offspring following either clinical CVS, or amniotic sac puncture at a corresponding stage, it is believed that an inadvertent rupture of the amnion during CVS may offer at least a partial explanation for the clinical findings. Human studies on these aspects are inevitably difficult, therefore, it seemed of value to reconsider the available experimental information which had been generated long before the CVS technique became available, and to develop a suitable murine model. It is planned, by using this model, to investigate the incidence, pattern, and type of disturbance and the possible underlying mechanisms which can be induced by CVS should an inadvertent puncture of the amniotic sac occur during the CVS procedure.

Table 1.1 Comparison of transcervical and transabdominal CVS (from Wapner, 1997; with permission)

	Transcervical	**Transabdominal**
Relative contraindications	Cervical polyps Retroverted uterus with posterior placenta Active cervical or vaginal herpes	Interceding bowel
Ease of learning	Learning curve required	Adaptation of amniocentesis technique
Sample size	Large sample, includes whole villi	Smaller sample, includes many small pieces
Patient discomfort	Minimal to absent	Moderate
Placental location	Better for posterior placenta	Better for fundal placenta

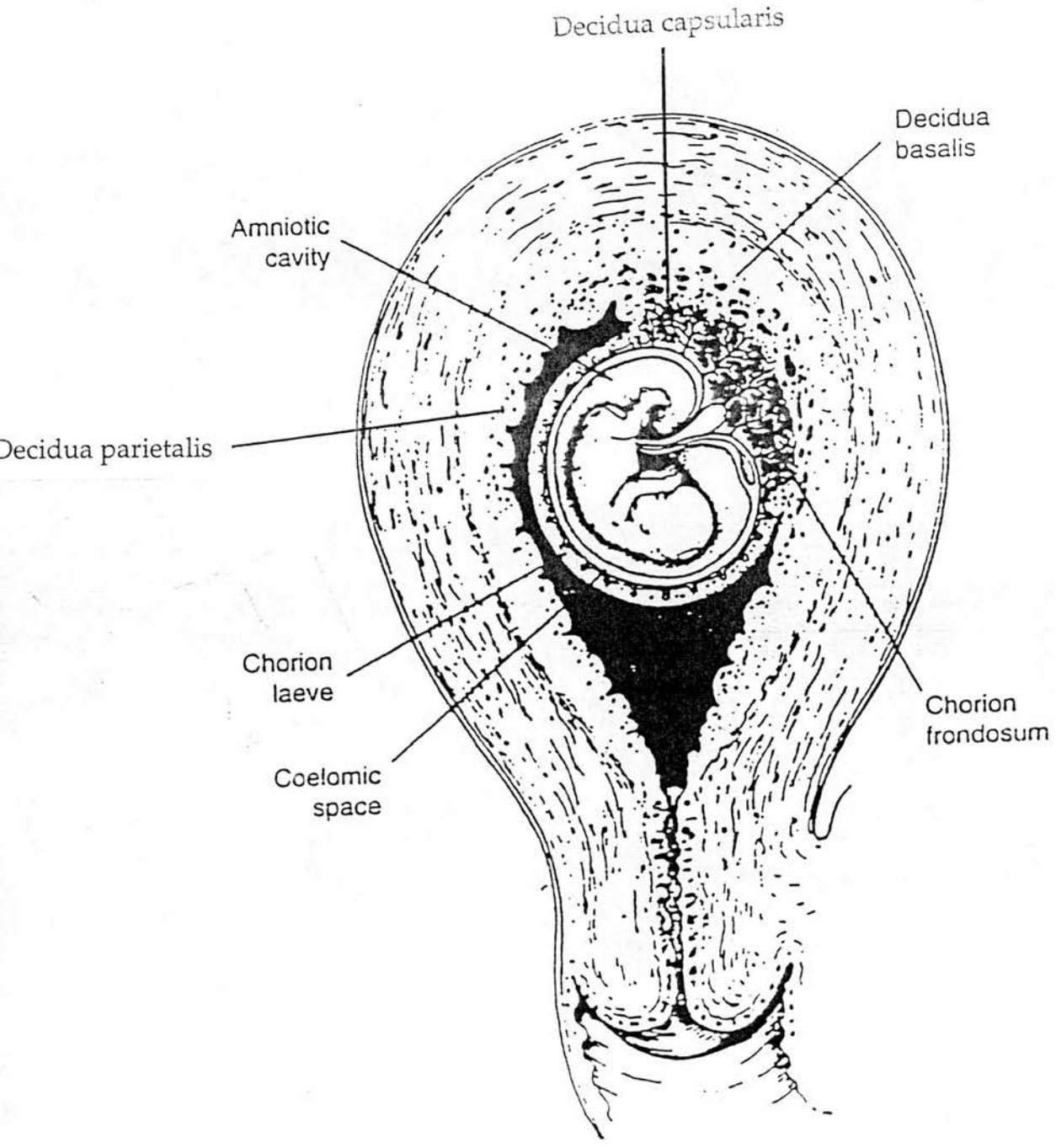


Figure 1.1 A schematic drawing of human intrauterine gestation between 9 to 12 weeks' pregnancy, during which time CVS is mostly carried out (from Wapner, 1997; with permission). The amniotic and the chorionic membranes as well as the tissues outside of the amniotic sac are shown. Note that the amnion and the chorion are not fused and are separated by a potential space (the coelomic cavity) during this period of development. Chorionic villus sampling is performed by inserting a catheter into the *chorion frondosum* where cells to be investigated are extracted from the site of insertion. It should be noted that since the vascular *decidua basalis* lies beneath the *chorion frondosum;* insertion of the catheter into this deeper level can lead to bleeding and the formation of haematoma.

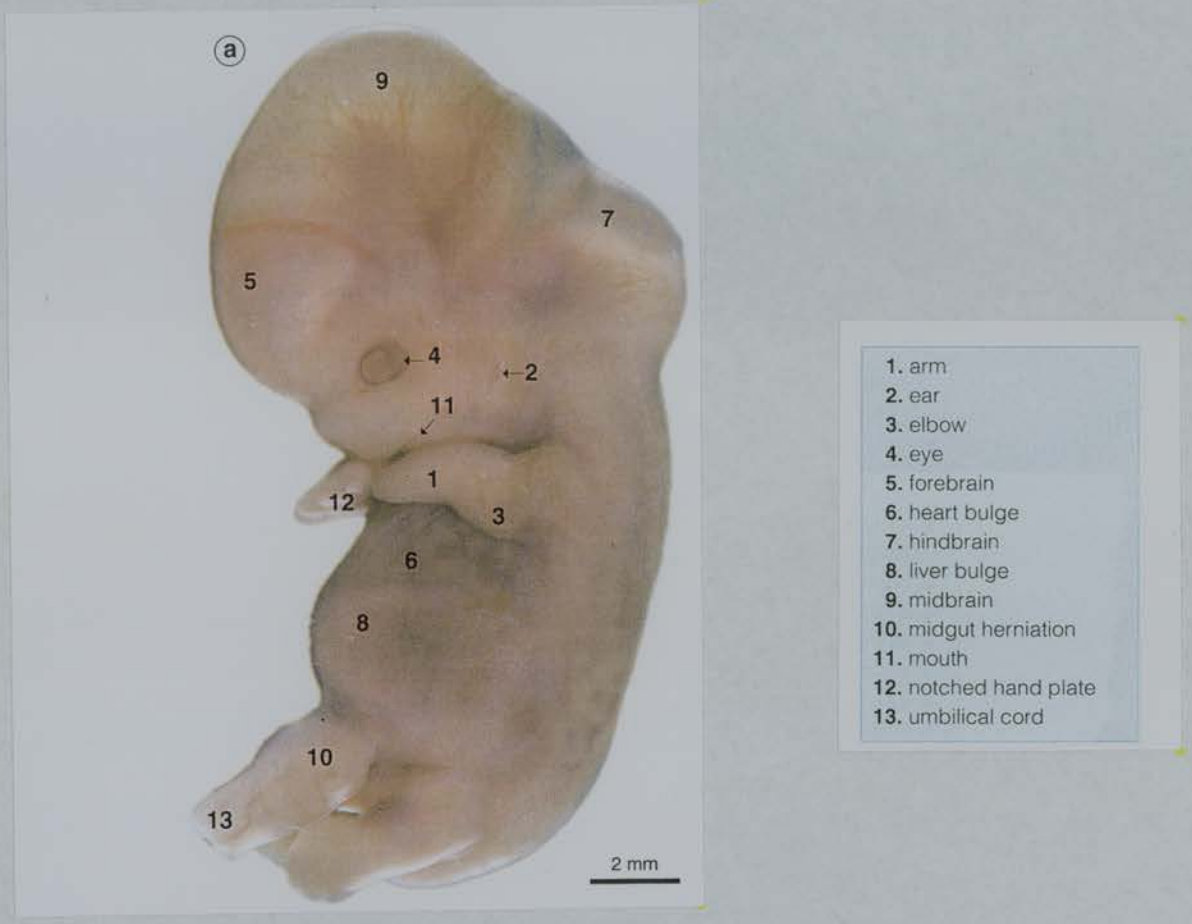


Figure 1.2. External morphology of developing human fetuses during 8 to 9 weeks of gestation (dated from last menstrual period; from England, 1996). At the end of week 6, the upper limb (forelimb) bud appears, and about 2 days later, the lower limb (hindlimb) bud appears. During week 7, the upper limb bud protrudes 90° form the body. During week 8, a paddle-shaped hand-plate forms distally in the forelimb. The grooves between the digital rays become deeper, making digital rays recognizable. During week 9, the lower limb forms a paddle-like foot-plate distally, and the grooves between each digital ray become visible.

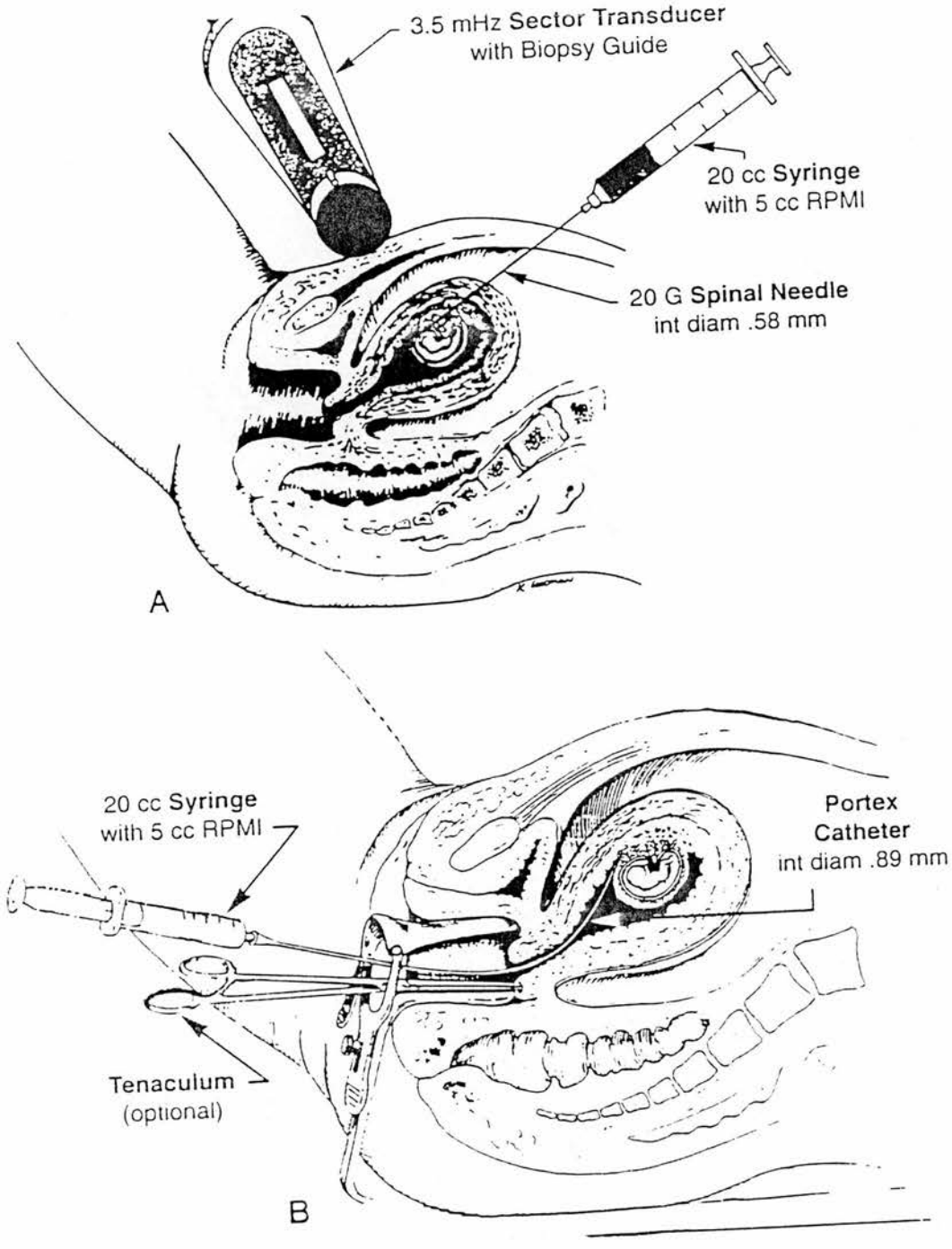


Figure 1.3 Transabdominal and transcervical CVS techniques (from Wapner, 1997; with permission). (A) Transabdominal CVS by a single needle. The needle tip is first inserted into the myometrium and then redirected parallel to the chorionic membrane with the aid of ultrasound guidance. Once the needle is within the placenta, the stylet is removed and 10 to 20 ml suction is applied with an attached syringe containing 5 mL of medium (for example RPMI medium). (B) Transcervical CVS is performed using a thin polyethylene catheter through which a stainless steel stylet has been inserted. The stylet fits through the catheter and both the polyethylene catheter and the stainless steel stylet have to pass through the cervix. They need to provide sufficient rigidity for adequate passage into the *chorion frondosum* without causing damage to the cervix. Prior to the performance of the CVS procedure, ultrasound scanning is applied to establish the site of the *chorion frondosum* and to confirm fetal viability. The relative position of the uterus and the cervix is identified, which allows catheter placement parallel to the chorionic membranes. Once the catheter is in place, the stylet is removed and approximately 10 to 20 ml of suction is applied with an attached syringe containing 5 ml of collecting medium.

Chapter 2: Establishment of an amniotic sac puncture (ASP) mouse model

2.1 Introduction

As indicated in chapter 1, the safety of first trimester CVS has been the major concern of clinicians who performed the procedure for prenatal diagnosis. A number of recent publications have raised the possibility of an association between first trimester chorionic villus sampling (CVS) carried out between days (d) 55 and 65 of gestation with a spectrum of abnormalities, including limb reduction defects and craniofacial abnormalities such as cleft palate, cleft lip, micrognathia and/or glosspotosis (Planteydt *et al.*, 1986; Christiaens *et al.*, 1989; Kaplan *et al.*, 1990; Editorial, 1991; Firth *et al.*, 1991a; Hsieh *et al.*, 1991; Mahoney, 1991; Mastroiacovo and Cavalcanti, 1991; Rodriguez and Palacios, 1991; Burton *et al.*, 1992, 1993; Froster and Baird, 1992; Kuliev *et al.*, 1992; Schloo *et al.*, 1992).

Many hypotheses have been proposed to explain the aetiology of these unexpected abnormalities. While the craniofacial defects may have a postural basis (see below, and also section 1.3.3.1), the majority of the authors have suggested a vascular etiology for the limb abnormalities observed in the CVS-exposed babies (Firth *et al.*, 1991b; Burton *et al.*, 1992; Brent, 1993; Report of the NICHHD Workshop, 1993). The following explanations have been proposed: (1) damage at the biopsy site might stimulate the secretion of vasoactive factors into the fetal circulation (2) fetal blood loss or the formation of thrombi within the vessels near the biopsy site might cause fetal hypotension, embolisation or hypoperfusion, resulting in anoxia of the extremities (Firth *et al.*, 1991b) (3) thrombus formation within the placental bed due to infection such as chronic villitis or basal deciduitis might induce ischaemia and necrosis in the distal limb areas (Hoyme *et al.*, 1982) (4) entrapment of one or more of the limb extremities into the obliterating extracoelomic gel might occur, leading to anoxia and hypoperfusion of the distal parts (Shepard *et al.*, 1991) (5) inadvertent rupture of the amnion during CVS might arise, followed by amniotic fluid leakage and intrauterine constraint, resulting in embryonic compression and anoxia in the distal limbs (Shepard *et al.*, 1991; Kaufman, 1994). Based on the clinical findings and results obtained from animal studies, the most plausible explanation for the abnormalities observed following CVS remains that unintentional pucture of the amnion occurs, and consequently induces the limb and craniofacial abnormalities (full details see section 1.3.3.4). It, therefore, became appealing to review animal studies with experimentally-induced puncture of the amnion, to investigate any abnormalities observed.

Concerns over the safety of amniocentesis were raised in the first report where this technique was sucessfully developed (Dewhurst 1956). In order to evaluate the safety of amniocentesis, Trasler and colleagues (1956) undertook a series of animal studies. A procedure of amniotic sac puncture (ASP) by a needle was used, allowing an uncontrolled leakage of amniotic fluid. These embryos were then

returned to the abdominal cavity and allowed to develop till autopsy. Out of the 17 treated embryos which survived till near term, 10 had a cleft palate, while a closed palate was present in all control embryos. The authors therefore proposed that amniotic sac puncture exerted a teratogenic effect on the developing palate in the mouse.

Similar observations were subsequently reported by Walker (1959) who attempted to investigate the underlying mechanism of ASP syndromes in a subsequent study. The aetiology of the cleft palate was suggested to be due to the compression by uterine muscles and extraembryonic membranes, as a result of reduction of hydrostatic pressure in the amniotic sac following ASP. An additional experiment to evaluate the teratogenic effect of extraembryonic membranes compression *alone* was performed in the same study. When the treated embryos and their associated membranes were released from the uterine horn and allowed to develop further till full term, only mild signs of compression, including cleft palate, were encountered and "*limbs twisted out of position*". The latter strongly implied that, following ASP, the compression either from the extraembryonic membranes alone or together with that from the uterine muscles was probably the major cause of cleft palate.

In the early rat ASP studies, limb abnormalities similar to those observed in the CVS-exposed infants were also reported (Poswillo, 1966; Poswillo and Roy, 1965; DeMyer and Baird, 1969; Love and Vickers, 1972; Singh and Singh, 1973; Singh *et al.*, 1974; Kino, 1975; Kennedy and Persaud, 1977; Houben, 1980; 1984; Houben and Huygens, 1987). Detailed analysis confirmed that vascular disruption in various forms was evident in those affected limbs.

It is worth noting that all these earlier animal ASP studies were performed at a developmental stage when CVS, instead of amniocentesis, would normally be performed clinically. For this reason, as well as possible inadvertent rupture of amnion during CVS, animal ASP models appeared to be suitable to investigate the association of CVS with those reported abnormalities, and to understand the underlying mechanisms. Clearly, the establishment of animal ASP models also has the advantage to overcome any ethical problem associated with human studies. Since mouse has been studied exclusively in our laboratory, it was important to establish a *mouse* ASP model in order to achieve the aims indicated above.

2.2 Materials and Methods

2.2.1 mouse

The mice used in this study were virgin (C57Bl x CBA) F_1 hybrid female mice aged between 8 to 10 weeks. The females were caged overnight with (C57Bl x CBA) F_1 hybrid males. In the following morning, the presence of a vaginal plug was taken as evidence of mating and the day was defined as day 1 (d1). Following mating, the females were kept under controlled environmental conditions (20° C; relative humidity approximately 50 %) and fed with a standard pelleted diet (Bantin & Kingman) with freely available water, until d 12, 13, 14, 15, and 16 of gestation.

2.2.2 Procedures of ASP

2.2.2.1 Preparation of anaesthesia (Avertin)

Avertin was prepared following the protocol described by Hogan *et al.* (1986). A stock of 100 % Avertin was prepared by mixing 10 g of tribromoethyl alcohol with 10 ml of tertiary amyl alcohol. The 100 % stock solution was kept wrapped in foil at 4° C. Shortly before use, the 100 % stock solution was diluted to 2.5 % in 0.9 % saline.

On d 12, 13, 14, 15, and 16 of pregnancy, the females were given a general anaesthetic, which was then followed by a midline laparotomy to expose the amniotic sacs for puncture. The females were weighed and injected intra-peritoneally with 0.016 ml of 2.5 % Avertin per gram of body weight. The anaesthetic usually took several minutes to be effective. The efficacy of Avertin was checked by the eye blinks and by reflex action when the mice were lightly pinched in their hindlimbs. Only fully-anaesthetized mice were operated on.

2.2.2.2 Midline laparotomy

A midline laparotomy was performed to expose the implantation sites. The abdomen of the females were first shaved and wiped with 70 % alcohol for sterilization. The body wall was opened (a 1.5 - 2.0 cm longitudinal incision) by cutting the skin with a pair of dissection scissors.

2.2.2.3 Amniotic sac puncture and post-puncture procedures

Following midline laparotomy, amniotic sac puncture was performed. No attempt was made to select either the right or left uterine horn for puncture. The more easily accessible horn was exteriorized on

each occasion and puncture was performed on that horn (defined as "experimental" horn). The ovary belonging to the "experimental" side, was identified, and the number and location of embryos and resorption sites recorded. The tip of a 21 or 25 gauge needle was employed to puncture through the wall of the "experimental" horns in the sites where each gestational sac was located. Care was taken to avoid the location of the placenta and major yolk sac blood vessels to reduce bleeding to a mimimum. Direct contact between the tip of the needle and the embryo was avoided during puncture. After puncture, the needle was then withdrawn, allowing an uncontrolled amount of amniotic fluid to leak out. Procedures were considered to be adequate only when withdrawal of the needle was followed by the escape of a small volume (estimated to be 1~2 ml) of amniotic fluid.

Once the puncture was completed, the uterine horn was replaced back in the abdomen and the peritoneum was sultured. The abdominal wall was closed with Michel clips and the mice allowed to recover in a warm environment without being disturbed. The females were then returned to their cages and kept under controlled environment (20° C; relative humidity approximately 50 %) and fed in a same way as it was following mating.

2.2.2.4 Dissection on day 19.0

The females were sacrificed on day 19 (d 19) of gestation by cervical dislocation. The uterine horns were removed and the fetuses were dissected free from their extra-embryonic membranes and transferred into phosphate buffered saline. All fetuses were immediately examined under a dissection microscope for evidence of morphological anomalies. Their crown-rump lengths (CR-lengths) were measured and the fetuses were then blotted free of surface saline and weighed. The fetuses were then sacrificed by chloroform inhalation and immersion fixed at room temperature in Bouin's fluid for 24 hours.

A representative selection of Bouin-fixed control and experimental fetuses were photographed to display the normal appearance of the limbs as observed in the control series, and the range of limb, tail and postural abnormalities observed in the experimental series.

2.2..2.5 Preliminary experiments on ASP procedure

To establish timing of amniotic sac puncture and optimal needle gauge which induces highest rates of palatal and limb abnormalities, preliminary experiments on ASP procedures were necessarily performed, involving a small number of mice being subjected to this procedure on d 12, 13, 14, 15 or 16 of gestation, with either 21 or 25 gauge needles.

2.2.2.6 Classification of the limb abnormalities

Classification of the limb abnormalities was difficult due to a multitude of descriptive terms with varying degree of specificity, in the literature (for example, see Hall *et al.,* 1962; Henkel and Willert, 1969; Forfar and Arneil, 1985). Following the proposal by Froster and Baird (1992), it was decided to adopt a classification based on suggestions of the American Society of Surgeons of the Hand and of the International Society of Prosthetics and Orthotics (Swanson, 1976). There was one abnormality observed, however, that did not correspond with any of the terms encountered. For this abnormality, the term *diastodactyly* was devised [from the Greek *diasta(nai)* to set apart + *daktyl(os)* finger], to cover those cases where there was lateral divergence of 2 adjoining digits. This condition contrasts with *ectrodactyly* where two digits may diverge because of the absence of all components of an intervening digit.

2.2.3 Statistics

Student's t tests and, where numbers of observations allowed, z tests were used to analyse the data.

2.3 Results

2.3.1 Determination of needle size and appropriate gestation stage for puncture

The results of preliminary experiments indicated that the earlier the amniotic sac puncture was performed during pregnancy, the higher rate of fetal mortality was found when assessed on d 19 of pregnancy. When amniotic sac puncture was carried out on d 12, only 6 % of fetuses survived. Even in the control series, although without being punctured, only about 50 % of fetuses survived following laparotomy at this stage of pregnancy. To produce the whole spectrum of palate and limb abnormalities, the critical gestation period for amniotic puncture procedure was found to be on d 13 and d 14. When this procedure was carried out on subsequent days (i.e. d 15 and d 16), no palatal defects were seen and the incidence of limb and tail abnormalities was low. Therefore, d 13 and d 14 were regarded as the appropriate gestation stage for puncture.

When a 25 gauge needle was used on d 13 and d 14, the incidence of abnormalities induced by the procedure was low. When amniotic sac puncture was carried out with a 21 gauge needle on either d 13 or d 14, however, a substantially higher incidence of abnormalities involving the palate, limb and tail were found without the induction of unacceptable level of embryonic resorption rates. The remaining experiments were, therefore, carried out using a 21 gauge needle, with a total of 21

pregnant females being operated on at d 13 of gestation, and a further 17 pregnant females on d 14 of gestation.

2.3.2 Findings following ASP at d 13 and d 14

The incidence of malformations induced by amniotic sac puncture carried out using a 21 gauge needle under general anaesthesia is listed in Table 2.1. For comparison, the incidence of malformations on d 13 and d 14 are listed together. The analysis was performed on d 19 of pregnancy. The percentages of abnormalities observed, as cited in the following text, refer only to those fetuses that survived to d 19. The abnormalities were divided into three principal categories: palate defect, limb abnormalities, and tail abnormalities, as can be seen in Table 2.1. In addition to those listed in Table 2.1, two other abnormalities occurred once each, namely exencephaly and omphalocele. Furthermore, general decreases of crown-rump lengths, and fetal weights were often found in the experimental series. The following sub-sections describe in detail all of the abnormalities observed.

2.3.2.1 Varying degrees of compression observed by external inspection of fetuses in the experimental series

External inspection of the fetuses in the experimental series revealed varying degrees of compression that were not found in the control series (Figures 2.1 and 2.2). In the most severe examples, compression caused evident wrinkling as well as 'moulding' of the head, body, limbs and tail. In some of the most severe cases, the compression made the fetuses ovoid in shape. The effect of compression, however, was not universal on all experimental series. In other cases, fetuses from the experimental series appeared morphologically to be apparently normal.

2.3.2.2 Survival rates

Fetuses subjected to amniotic sac puncture on d 13 exhibited a resorption rate of 47 % (see Table 2.1) which was much higher than 19 % resorption rate observed when this procedure was carried out on d 14. In the control series, 97 % of the d 13 and 90 % of the d 14 controls survived when examined on d 19. None of the controls that survived displayed any gross abnormalities. Of the experimental fetuses that survived, 70 % of those from the d 13 series exhibited at least one abnormality, whilst 48 % of those treated on d 14 exhibited at least one abnormality.

2.3.2.3 Palate defects

Cleft palate was present in 35 % of fetuses operated on d 13, whilst the incidence was in 27 % of those that had been operated on d 14. By dissecting the mandible and the tongue free, a complete cleft of

the posterior palate was observed. Furthermore, the tongue showed evidence of compression, consistent with the shape of the cleft. In all cases in which palatal defects were found, the primary palate was seen to be intact.

2.3.2.4 Limb abnormalities

The limb abnormalities observed following amniotic sac puncture included adactyly (lack of digits), syndactyly (fusion of digits), synonychia (fusion of nails), brachysyndactyly (fusion and shortening of digits), diastodactyly (lateral divergence of two adjacent digits), ulnar or radial deviation of the wrist, talipes equinovarus and fusions of a limb to another part of the fetus. A representative example of each of these abnormalities is presented in Figure 2.3 (at the end of this chapter), and appropriate descriptions given in the figure legend. The relative frequencies of each of these abnormalities are detailed in Table 2.2. Syndactyly and brachysyndactyly (mutually exclusive) and talipes equinovarus were the most frequent abnormalities produced, with an incidence of 37, 33 and 20 % in d 13 fetuses, respectively, and incidences of 36, 20 and 45 % in d 14 fetuses, respectively.

It was noted that the hindlimbs displayed higher incidence of abnormalities than the forelimbs ($P < 0.01$) (see Table 2.1). This was particularly evident when both the right and the left hindlimb were examined. It was noted that they displayed more abnormalities when compared with the right and the left forelimbs, and a similar situation applied to both the d 13 and d 14 series.

2.3.2.5 Tail abnormalities

Varying degrees of abnormal curvature and transverse reduction deformities were found in the fetal tails in the experimental series (see Table 2.1). No tail abnormalities were found in the control series. Tail abnormalities were also proved more commonly encountered in d 13 compared with d 14 fetuses (43 % compared with 19 % ($P < 0.01$)). Representative examples of the tail abnormalities are shown in Figure 2.4.

2.3.3 Detailed data analyses on the findings following ASP carried out either on d 13 or d 14

As mentioned, three principal categories of abnormality were found following amniotic sac puncture at d 13 and d 14 when fetuses were analysed on d 19. When the three principal categories of abnormality were considered, it became apparent that most fetuses with cleft palate had an additional defect. Frequencies of combination of the 3 principal categories of abnormality are presented in Table 2.3.

When the crown-rump length and the weight of the fetuses were considered, data analysis revealed significant differences between the control and experimental fetuses in both the d 13 and d 14 series ($P < 0.01$), with the experimental embryos being significantly lighter and smaller than the controls. Furthermore, differences were found between the crown-rump lengths of the d 13 fetuses that had one or more abnormality, and those experimental fetuses that appeared on gross inspection to be completely normal ($P < 0.01$). Similarly, the weight of the apparently normal and abnormal d 14 experimental fetuses differed significantly ($P < 0.05$) (see Table 2.4). A summary of the significant differences calculated is presented in Tables 2.5 and 2.6.

When the results of d 13 and d 14 were pooled, 69 out of 121 (57 %) fetuses showed at least one abnormality. Although the critical period in rats is d 15 (normal rat gestation ~ 22 d), this is equivalent, in developmental terms, to d13/d14 in the mouse (Witschi, 1962; Kaufman, 1992). The incidence of palatal, tail and most types of limb defect, with the exception of diastodactyly (which showed equal incidence), was higher on d 13 than d 14 (overall incidence 61 %), compared with 39 % ($P < 0.01$), respectively).

2.4 Discussion

2.4.1 Hypothesis regarding the possible mechanisms involved in the abnormalities following ASP

There are at least two major mechanisms that are likely to cause the abnormalities observed following amniotic sac puncture -- intrauterine constraint and vascular interruption, although, clearly it is only very rarely possible to establish unequivocally what the underlying cause(s) of a particular abnormality may be (Love and Vickers, 1972; Kino, 1975; Kennedy and Persaud, 1977; Houben, 1984; Kaufman, 1994; MacIntyre *et al.*, 1995).

2.4.1.1 Limb abnormalities

2.4.1.1.1 Intrauterine constraint

Intrauterine constraint had been proposed by Poswillo (1966, 1968) and later by Miller (1983). Using a rat amniotic sac puncture model, Poswillo (1966) reported that limb deformities were found in 29 % of rat embryos exposed to ASP. The limbs were compressed into abnormal positions and often displayed abnormalities such as ring constrictions, syndactyly, and phocomelia (Poswillo, 1966). Poswillo (1966) noted that, at the stage when amniotic sac puncture was carried out, limb bud development had been fully established, "*even to differentiation into upper and lower limb segments and digits*". He concluded that "*the causal mechanism has been one of intrauterine focal necrosis*

with subsequent repair, and not one of arrested development". Similar results were also reported by Miller (1983) who also proposed an intrauterine constraint mechanism.

The intrauterine constraint mechanism appears to help explaining the different incidence of limb abnormalities between the forelimbs and the hindlimbs (see section 2.3.2.4 and table 2.1) in several ways: if the limbs are affected by direct pressure, it is possible that the forelimbs are 'protected' to some extent because of their anatomical position, i.e. being located in close proximity to the overhanging fetal head (Kennedy and Persaud, 1977; MacIntyre *et al.,* 1995). Furthermore, if the limbs have been affected by the intrauterine constraint and with induced focal necrosis with subsequent repair as proposed by Poswillo (1966), one should find that the d 13 series would display more abnormalities in the limbs than the d 14 series, which is shown by the data in the present study. The fact that the forelimbs are about 12 hours more advanced developmentally than the hindlimbs at about d 12.5 - 13 in the mouse also helps to explain the higher incidence found in the hindlimbs, in that the hindlimbs, being less well developed structures, are likely to be more sensitive to teratogenic stimuli (MacIntyre *et al.,* 1995).

The causes of intrauterine constraint are probably three-fold. Firstly, the uterine blood vessels, like all other blood vessels, exert an immediate reaction to trauma by instant constriction which also results in muscles constriction. Although the instant constriction is temporary and is not likely to be the major cause of 'intrauterine constraint', the compression is initiated. Secondly, and probably of greater importance, the procedure of amniotic sac puncture in this study was considered to be adequate only when an uncontrolled amount of amniotic fluid leakage occurred (see section 2.2.2.3). The loss of amniotic fluid will inevitably alter the pressure in the intra-amniotic compartment and such alteration is unlikely to be alleviated shortly after puncture. The result is a slump of uterine membranes for at least a certain period of time, which is enough to cause intrauterine constraint and subsequently contributing teratogenic stimuli to embryos at gestation days 13 and 14. Thirdly, the traumatized uterine blood vessels probably released vasoactive amines or peptide factors from their endothelial cells that contribute to the constriction of the uterine membranes.

2.4.1.1.2 Circulatory disturbance

In addition to the intrauterine constraint noted above, the vascular interruption had also been observed following experimental amniotic sac puncture (Houben , 1984) and had been indicated as a potential cause for limb abnormalities (Kaufman, 1994). The vascular interruption is probably due to the effect of venous stasis, hypervolaemia, and localized hypoxia (Houben, 1984); all are potential risk factors for the limb deformities (Kaufman, 1994). For example, hypoxia had been implicated in the mechanism of apoptosis (Arend and Wyllie, 1991) which, as current opinions generally agree, is implicated in the control of limb morphogenesis.

Another observation that may complicate vascular interruption is haemorrhage. Haemorrhage can occur from the uteroplancental circulation as well as from the embryo itself. The procedure of puncture inevitably causes a limited degree of haemorrhage, at the site of puncture. Once haemorrhage has taken place, as described in the preceding section 2.4.1.1.1, vasoactive factors will be released to affect the uterine membrane as well as the embryo. Houben (1984) described haemorrhage in rat tails shortly after amniocentesis, although it is of interest that he stated that '... *haemorrhage can be extensive and never impairs development.*' In the present study, however, haemorrhage was observed mostly in the developing forelimbs and hindlimbs, implying more direct involvement in the pathogenesis of limb abnormality than Houben's observation (1984). This will be described and discussed in chapter 5.

2.4.1.2 Palate defects and tail abnormalities

Palate defects were found in about one third of the experimental series, being 35 % of d 13 and 27 % of d 14 (see Table 2.1). The cause of the palate defect found in this study is probably mainly due to the effect of compression due to intrauterine constraint, although an indirect involvement of the vascular interruption cannot be excluded. Since no haemorrhage or other evidence of vascular interruption have been observed in the palatal region, neither in previous reports nor in the present study. Additionally, as indicated previouly in section 1.3.3.1, obvious evidence of compression were observed in the craniofacial region in the earlier animal ASP studies (Poswillo, 1966; Walker, 1959; 1967; 1968a). For the tail abnormalities, however, haemorrhage had been observed in the rats subjected to experimental amniocentesis (Houben 1984). On this basis, vascular interruption, therefore, is likely to be involved in the pathogenesis of tail abnormalities.

2.4.1.3 Reduced crown-rump length and weight

Both intrauterine constraint and vascular interruption explain the reduced fetal crown-rump length and weight observed in the experimental series following amniotic sac puncture. The general compression exerted by intrauterine constraint can potentially inhibit the growth and axial extension of fetuses. On the other hand, any disturbance in fetal circulation could affect the supply of nutrition and oxygen; both are critical in the fetal growth and hence are important in determining the fetal weight.

2.4.2 Comparisons between mouse ASP and early human CVS

2.4.2.1 Procedures and gestation periods during mouse ASP and human CVS

Animal ASP studies involve a puncture through the chorionic and amniotic membranes, as well as through the uterine muscles. Additionally, in either the rat or mouse ASP, a midline laparatomy was performed for easy access to the amniotic sacs. While in *standard* human CVS, transabdominal approach requires the insertion of a needle through maternal abdominal wall and the uterine muscle, and transcervical method requires the insertion of the needle through the cervix to the biopsy site. Neither human CVS procedures are performed with the puncture of the amnion in an ideal practice. However, when the inadvertent puncture of the amnion occurs during CVS, this mouse model very closely resembles the clinical situation.

In this study, the incidence of palate, limb and tail abnormalities was greater when amniotic sac puncture was carried out on d 13 compared with d 14 of pregnancy. It is worth noting that the limb abnormalities associated with CVS appeared to show a similar degree of temporal specificity: 56 d - 66 d in one study by Firth *et al.* (1991), and 63 d - 79 d in another report by Burton *et al.* (1993). The stage of development of mouse embryos at the time of amniotic sac puncture in the present study is equivalent to about 50 - 60 d postovulation, or Carnegie stages 20 - 23 of human embryonic/fetal development (O'Rahilly and Müller, 1987; Kaufman, 1992). These findings may indicate that a common causal mechanism is involved (MacIntyre *et al.*, 1995).

2.4.2.2 Fetal and placental development during ASP in the mouse and CVS in the human

During the period of development when mouse ASP or human CVS is performed, similar fetal development can be seen externally.

The craniofacial region constitutes almost half of the crown-rump length in the human fetus at the 9th week, which is also characterized in the mouse fetuses at d 13 - 14. Such a high craniofacial proportion in relation to the whole embryonic body implies that, should there be any intrauterine constriction, the craniofacial region and the developmental events therein will be exposed to the greatest effect of compression, particularly the event of palatal closure that takes place between 8 - 10 weeks of gestation.

Comparable features of limb development are also seen during the period between the mouse ASP and the human CVS procedures. In the human, the upper limb (forelimb) first appears late in the 6th week of pregnancy (dated from the last menstrual period), while the lower limb (hindlimb) appears

two days later (England, 1996). This cephalo-caudal maturity gradient is retained throughout development and is also seen in the mouse. In the mouse, the forelimbs are about 12 hours more advanced in development than the hindlimbs at around d 12.5 - 13 (Kaufman, 1992). The elbow has already moved 90 degrees dorsally at weeks 9 - 11 (dated from the last menstrual period), with fingers or toes produced in the 8 th week of gestation in the human (England, 1996), while in the mouse at d 13 of gestation the palmar surfaces of the handplates are directed inferomedially and the definitive location and width of the digits are clearly seen (Kaufman, 1992). In the *mouse*, however, the webbing seen in the region of the interdigital zones is a characteristic feature of development at E 12.5-13 (Kaufman, 1992) when the experiments reported in this thesis were done, and equivalent to the interdigital development seen in the human at about 8.5 weeks (i.e. about 60 days after the last menstrual period). CVS was *formally* performed as early as 55-65 days after the last menstrual period (i.e. from about 41-51 days post-conception), but this procedure is now performed a week later because of recent WHO guidelines (Kuliev, *et al.*, 1996). This additional week means that the procedure done at a more mature state of limb development and a far lower frequency of limb abnormalities now results.

Comparable features of development can also be seen in the placenta. In the human, the chorionic villi start to be perfused by the fetal circulation at the 6th week of gestation and by the 9th week of gestation fetoplacental and uteroplacental circulations have already been established (Kaufmann and Burton, 1994; Kingdom and Sibley, 1996). The comparable establishment of fetoplacental and uteroplacental circulations are necessarily present in the mouse to support the fetus which is characterized by similar external features to those seen in human fetuses. Thus, if any structural or functional abnormalities are induced by the procedures of ASP/CVS, comparable effects may be expected to be seen in both the human and the mouse. It is also worth noting that the placenta is an endocrine organ of considerable capacity and diversity. Steroid hormones such as oestrogens and progesterone as well as polypeptide hormones like chorionic gonadotrophin and placental lactogen are readily produced by the placenta during pregnancy. Any traumatic effect on the placenta during the procedures of ASP/CVS may therefore affect the subsequent development of endocrine functions, and thus affect the general development of the fetus.

2.4.2.3 Amniotic fluid leakage following ASP in the mouse and CVS in the human

Amniotic fluid leakage differs in the mouse ASP and human CVS in two respects: the incidence and the relative proportion of leakage in relation to the total volume of amniotic fluid in the amnion. Leakage of amniotic fluid is believed to occur in at least 1 - 2 % of all cases of amniocentesis carried out during the second trimester of pregnancy for the purpose of genetic analysis (Crane and Rholand, 1986). The leakage, however, is said to resolve spontaneously within 48 hours (Crane and Rholand, 1986). The overt leakage of amniotic fluid following CVS has occasionally been reported. For

example, Hogge *et al.* (1986) reported that in their study of 1000 cases of CVS, the incidence of amniotic fluid leakage was 0.2 %. The overall incidence of amniotic fluid leakage in the MRC trial was 0.43 % (MRC Working Party, 1991; Editorial, 1991). For the mouse ASP, since uncontrolled amniotic fluid leakage was set as a guideline of completion of the procedure (see section 2.2.2.3), leakage was specifically induced in the mouse model.

The relative proportion of leakage in relation to the total volume of amniotic fluid is believed to be much higher in the mouse than in the human, based on the much smaller size of mouse amnion in comparision to that in the human. Such a higher proportion of leakage in the mouse ASP model is likely to have more impact on the fetus and the membranes as well.

2.4.2.4 Types and incidences of abnormalities following ASP in the mouse and CVS in the human

Types of abnormalities observed following ASP in the mouse is similar, as demonstrated in the present study, in the range of abnormalities, namely the oromandibular-limb hypogenesis spectrum, following CVS in the human. This coincidence reflects well the morphological and physiological comparability between the mouse and the human, as well as the comparability between ASP and CVS in terms of the procedures involved and the gestation stage when these procedures are performed. The incidence of abnormalities following ASP in the mouse is certainly much higher than those in the human following CVS. This is, however, purposely induced in order to see the maximum incidence in the animal model.

2.5 Perspectives provided by the mouse ASP model

Clearly, it is only rarely possible to establish unequivocally an animal model with its corresponding human condition, either for a genetic disease or an induced teratogenesis. This has always posed questions on whether the particular model employed is sufficiently closely related to the human conditions to an extent that it can be regarded as authentic. The authenticity is sometimes achieved without much controversy. Most of the time, however, to establish an animal model demands an open debate without a subsequent conclusion. This is due to many inherent differences between animals and human beings, in terms of their characteristics in body structures, genome sizes, and in their biochemical and physiological activities. However, we believe that the teratogenic effects of mouse amniotic sac puncture presented in this study in relation to the human CVS-induced abnormalities is a valid one. Also, the abnormalities observed in this study provides a model of teratogenic syndrome and useful insights into the normal development in the limb, palate, and tail.

Day of pregnancy	Group	Total pregnant females	Total implants analysed	Total viable fetuses (%)	Total normal fetuses (%)	Palate defect	Limb abnormalities				Tail abnormalities (%)
							Forelimb		Hindlimb		
							Right (%)	Left (%)	Right (%)	Left (%)	
13	Experimental*	21	102	54 (53)	16 (30)	19 (35)	12 (22)	11 (20)	19 (35)	25 (46)	23 (43)
	Control†		86	83 (97)	83 (100)	—	—	—	—	—	—
14	Experimental*	17	83	67 (81)	36 (54)	18 (27)	7 (10)	5 (7)	15 (22)	17 (25)	13 (19)
	Control†		61	55 (90)	55 (100)	—	—	—	—	—	—

* Exposed to amniotic sac puncture; † embryos from contralateral uterine horns.

Table 2.1 Results of abnormalities induced by amniotic sac puncture carried out under general anaesthesia on either day 13 or day 14 of pregnancy. The puncture was carried out by passing a 21 gauge needle through the wall of the uterus in the location of each gestational sac, with care being taken to avoid damage to the placenta and the major blood vessels associated with the yolk sac. Following the puncture, an uncontrolled amount of amniotic fluid was allowed to escape from the amniotic sac. The amniotic sacs in the contralateral side were untouched, acting as controls. The uterine horn was then replaced in the abdomen and the peritoneum sutured. The females were sacrificed on day 19 of gestation. The embryos were analysed and abnromalities were recorded and classified according to their gross morphology.

Day of pregnancy		Limbs affected (%)	Adactyly	Syndactyly	Synonychia	Brachy-syndactyly	Diasto-dactyly	Ulnar/radial deviation	Talipes equinovarus	Fusion of limb to other parts
13	LFL	11 (16)	2	4	0	2	0	2	N/A	1 (tail)
	RFL	12 (18)	0	4	1	3	0	5	N/A	0
	LHL	25 (37)	1	10	2	7	3	N/A	6	1 (tail)
	RHL	19 (28)	0	7	3	10	1	N/A	3	0
	Total	67	3	25	6	22	4	7	9	2
	%		4	37	9	33	6	30	20	3
14	LFL	5 (11)	0	2	0	2	2	0	N/A	1 (neck)
	RFL	7 (16)	0	4	0	1	1	0	N/A	1 (neck)
	LHL	17 (39)	0	7	0	4	1	N/A	9	0
	RHL	15 (34)	0	3	0	2	0	N/A	11	0
	Total	44	0	16	0	9	4	0	20	2
	%		0	36	0	20	9	0	45	5

LFL, left fore limb; RFL, right fore limb; LHL, left hind limb; RHL, right hind limb; *multiple abnormalities in each limb have been counted separately.

Table 2.2 Detailed analysis of the varieties of abnormalities* observed in 67 affected limbs of mouse fetuses subjected to amniotic sac puncture on the 13th day of gestation, and 44 limbs of mouse fetuses subjected to amniotic sac puncture on the 14th day of gestation. Fusion of a limb to another part of the fetus, either its tail or neck, is also listed. The classification of limb abnormalities was based on the suggestions of the American Society of Surgeons of the Hand and of the International Society of Prosthetics and Orthotics (Swanson, 1976). One abnormality, however, did not correspond with any of the terms suggested by the two societies mentioned. A term, *diastodactyly*, was coined [from the Greek *diasta(nai)* to set apart + *daktyl(os)* finger] to described those cases where lateral divergence of 2 adjoining digits was found.

Day of pregnancy	Viable fetuses (%)	CP only	LA only	TA only	CP+TA	CP+LA	LA+TA	CP+LA+TA
13	54 (53)	3	8	2	0	4	9	12
14	67 (81)	3	8	1	1	7	4	7

CP, cleft palate; LA, limb abnormality; TA, tail abnormality.

Table 2.3 The spectrum of combined abnormalities found in the palate, the limb, and the tail in embryos subjected to amniotic sac puncture on either the 13th or the 14th day of gestation. The embryos were analysed on day 19 of gestation, with their gross morphological abnormalities in recorded.

Day of pregnancy		Normal* Exp.		Abnormal† Exp.		All Exp.		Control	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
13	C–R	21.53	0.374	20.41	0.288	20.74	0.241	22.67	0.165
	Wt	1.072	0.019	1.069	0.021	1.070	0.016	1.133	0.011
	No.	16		38		54		83	
14	C–R	21.88	0.258	21.20	0.353	21.57	0.217	23.31	0.162
	Wt	1.150	0.013	1.084	0.031	1.120	0.016	1.203	0.013
	No.	36		30‡		66‡		55	

* No abnormal features present; † one or more abnormal features present; ‡ one exencephalic fetus excluded from the analysis. C–R, crown–rump length (mm); Wt, weight (g); S.E.M., standard error of the mean; Exp., experimental.

Table 2.4 Data of C-R (crown-rump) lengths and weights of fetuses subjected to amniotic sac puncture on either the 13th or the 14th day of gestation. The embryonic lengths and weights were measured on day 19 of pregnancy. Only data from embryos that were viable at the time of measurement were included.

Day of pregnancy		Abnormal	Normal	All experimental	Control
13	Abnormal*	—	$P < 0.01$	—	$P < 0.01$
	Normal†	$P < 0.01$	—	—	$P < 0.01$
	All experimental	—	—	—	$P < 0.01$
	Control	$P < 0.01$	$P < 0.01$	$P < 0.01$	—
14	Abnormal	—	$\underline{P > 0.05}$	—	$P < 0.01$
	Normal	$\underline{P > 0.05}$	—	—	$P < 0.01$
	All experimental	—	—	—	$P < 0.01$
	Control	$P < 0.01$	$P < 0.01$	$P < 0.01$	—

The underlined P values indicate that no significant difference was found at the 5% level. * One or more abnormal features present; † no abnormal features present.

Table 2.5 Significance values for crown-rump length data for day 13 and day 14 series. The significance values were derived from student's t tests. When the numbers of observations allowed, the significance values were derived from z tests.

Day of pregnancy		Abnormal	Normal	All experimental	Control
13	Abnormal*	—	$\underline{P > 0.05}$	—	$P < 0.01$
	Normal†	$\underline{P > 0.05}$	—	—	$P < 0.01$
	All experimental	—	—	—	$P < 0.01$
	Control	$P < 0.01$	$P < 0.01$	$P < 0.01$	—
14	Abnormal	—	$P < 0.05$	—	$P < 0.01$
	Normal	$P < 0.05$	—	—	$P < 0.01$
	All experimental	—	—	—	$P < 0.01$
	Control	$P < 0.01$	$P < 0.01$	$P < 0.01$	—

The underlined P values indicate that no significant difference was found at the 5% level. * One or more abnormal features present; † no abnormal features present.

Table 2.6 Significance values for fetal weight data for day 13 and day 14 series. The significance values were derived from student's t tests. When the numbers of observations allowed, the significance values were derived from z tests.

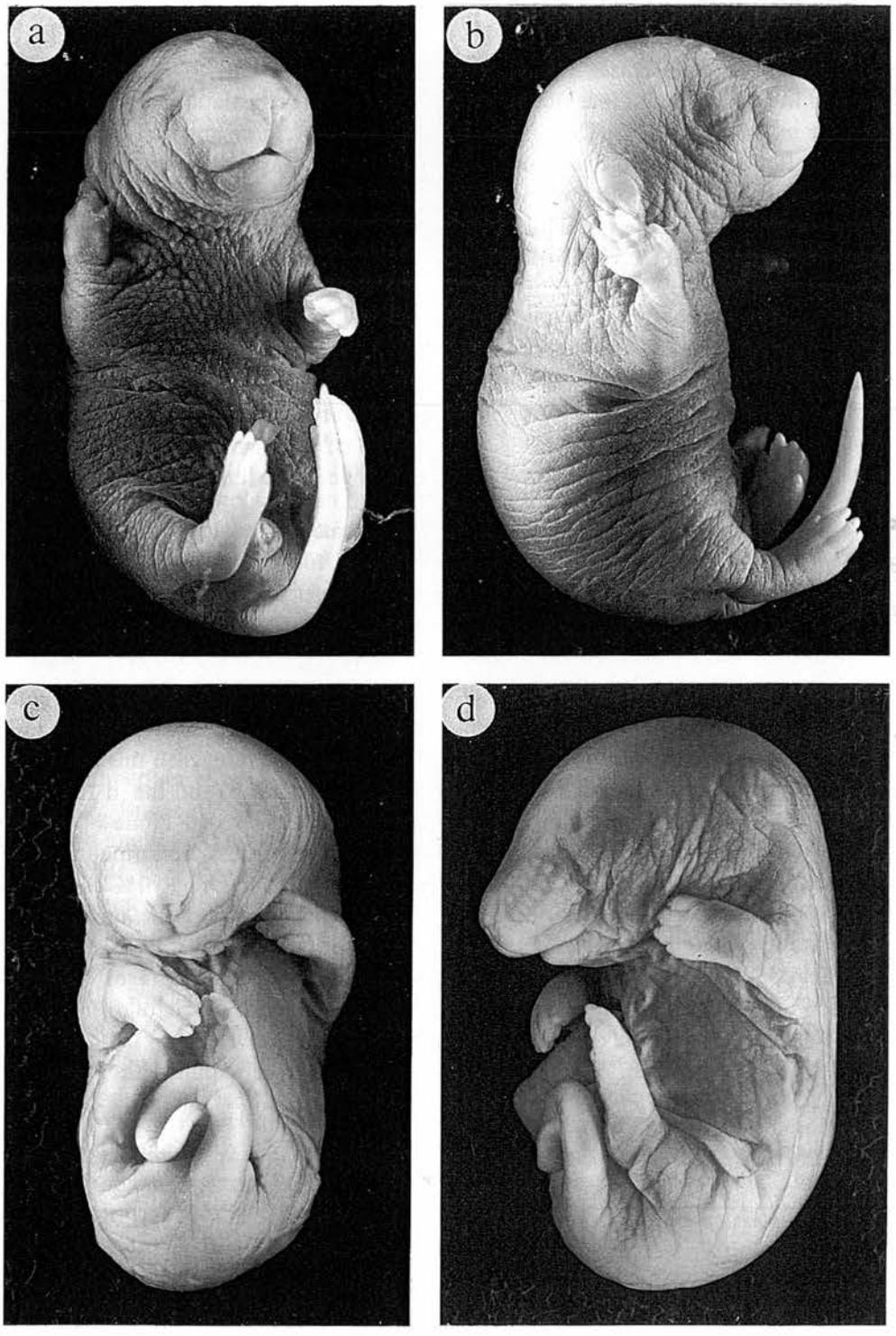

a
b
c
d

Figure 2.2 Moderate to severe and severe degree of compression is observed in two experimental fetuses isolated on d 19 of gestation. Both have an abnormally deficient lower jaw profile suggesting contact under pressure with the thoracic wall. In addition, both fetuses display an ovoid profile and tail deformities. The skin is considerably wrinkled in both fetuses. Frontal (a) and lateral (b) views of a fetus showing a moderate to severe degree of compression, with a transverse reduction deformity of the tail. The forelimb presents syndactyly and synonychia of digit 3 and 4. Mild talipes equinovarus is observed in both hindlimbs. Frontal (c) and lateral (d) views of a fetus showing a severe degree of compression. Extremely diminished lower jaw is observed. A severe degree of craniocaudal compression is also evident. Note particularly the abnormal curvature and "kinked" deformity of the tail. Syndactyly is found involving digits 2 and 3 of the right forelimb. Marked talipes equivarus is detected in both hindlimbs. Approximate magnification x4.

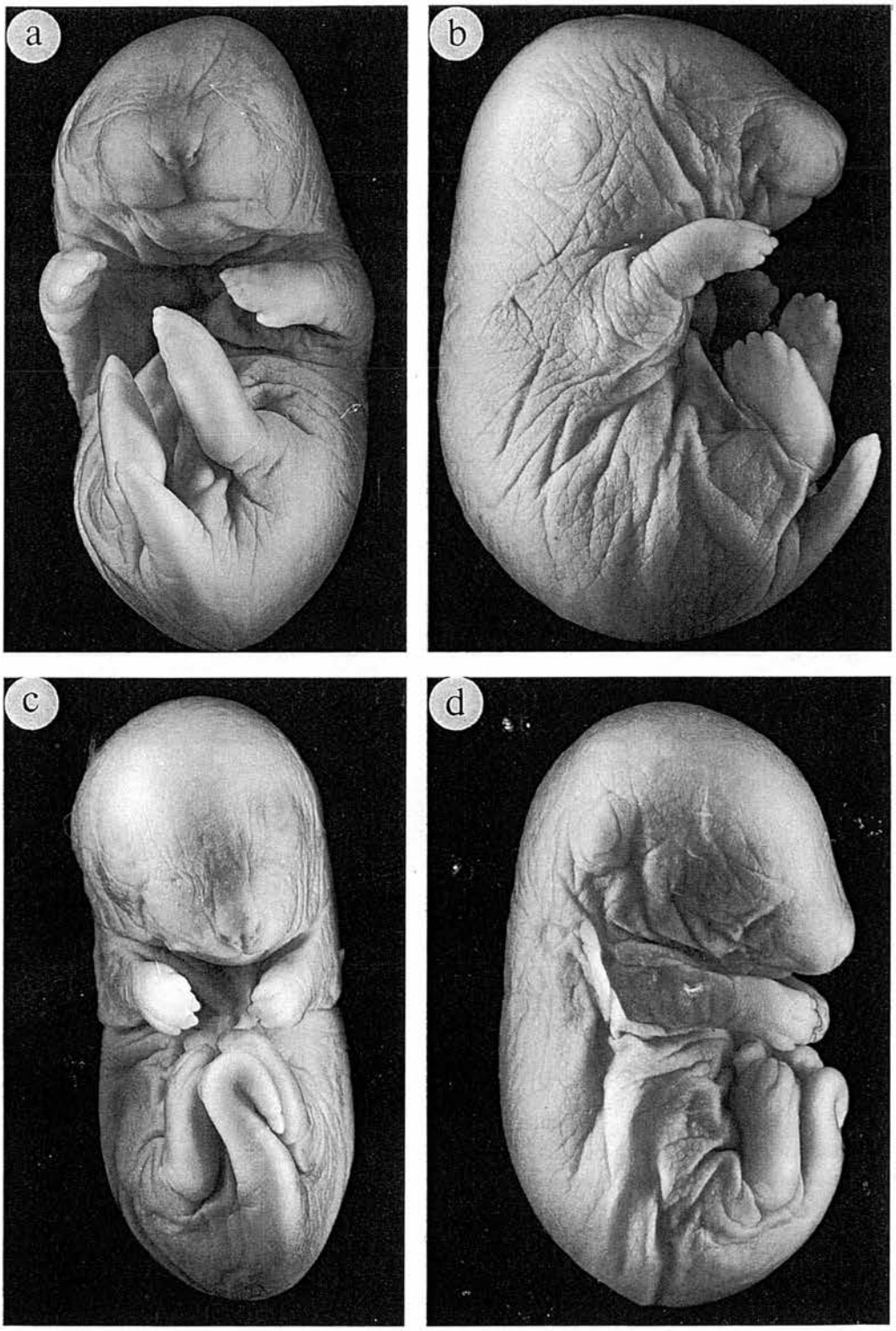

a
b
c
d

Figure 2.3 Variety of limb deformities observed in experimental fetuses isolated on d 19 of gestation.

(a) Adactyly. Lack of digits A dorsal aspect of hindlimb shows no recognisable digits, but with little sign of small nail rudiments.

(b) Syndactyly. Fusion of digits A dorsal aspect of hindlimb to show syndacyly

(c) Brachysyndactyly. Fusion and shortening of digits A dorsal aspect of hindlimb shows brachysyndactyly.

(d) Synonychia (fusion of nails) and syndactyly A dorsal aspct of a forelimb displaying both synonychia and syndactyly.

(e) Diastodactyly. Lateral divergence of two adjacent digits A plantar view of hindlimb to show diastodactyly involving digits 2 and 3.

(f) Radial deviation A dorsal view of forelimb to display radial deviation.

(g) Diastodactyly and syndactyly A dorsal aspect of hindlimb demonstrating both diastodactyly and syndactyly.

(h) Plantar view of hindlimb illustrated in (g) showing evidence of potential amniotic band (involving digit 1).

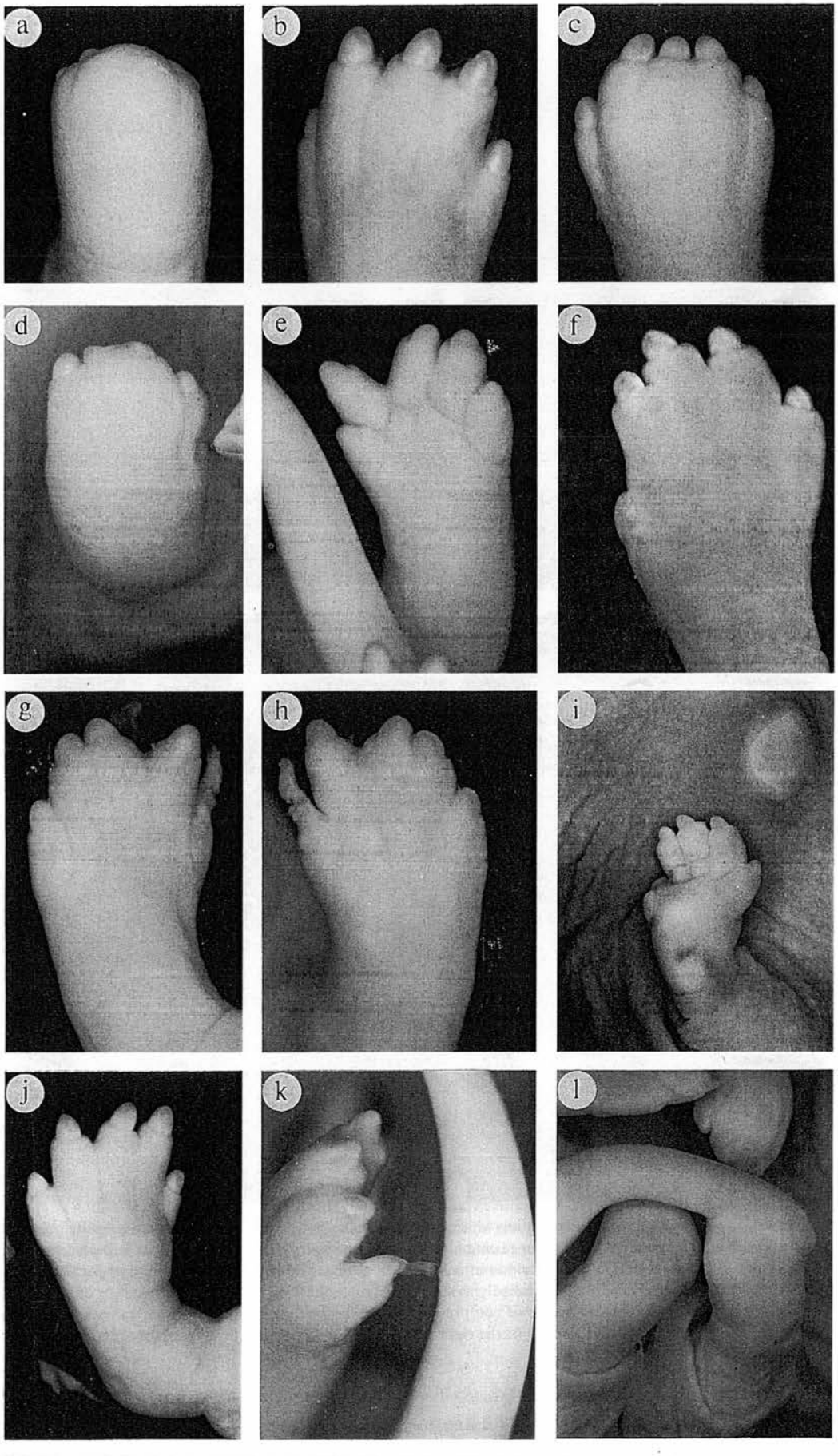
(i) Fusion of limb to side of neck, just caudal to left ear; a lateral view.

(j) Talipes equinovarus. Medial deviation of paw region, making the lateral border of the paw directed inferiorly; a lateral aspect.

(k) Fusion of hindlimb to tail by thin band; a frontal view

(l) Fusion of left forelimb to tail; a frontal view. Note the rostral part of the fetus is directed to the right of the photograph.

Approximate magnification: x 10


a
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e
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l

Figure 2.4 Spectrum of tail abnormalities noted in experimental fetuses isolated on d 19 of gestation.

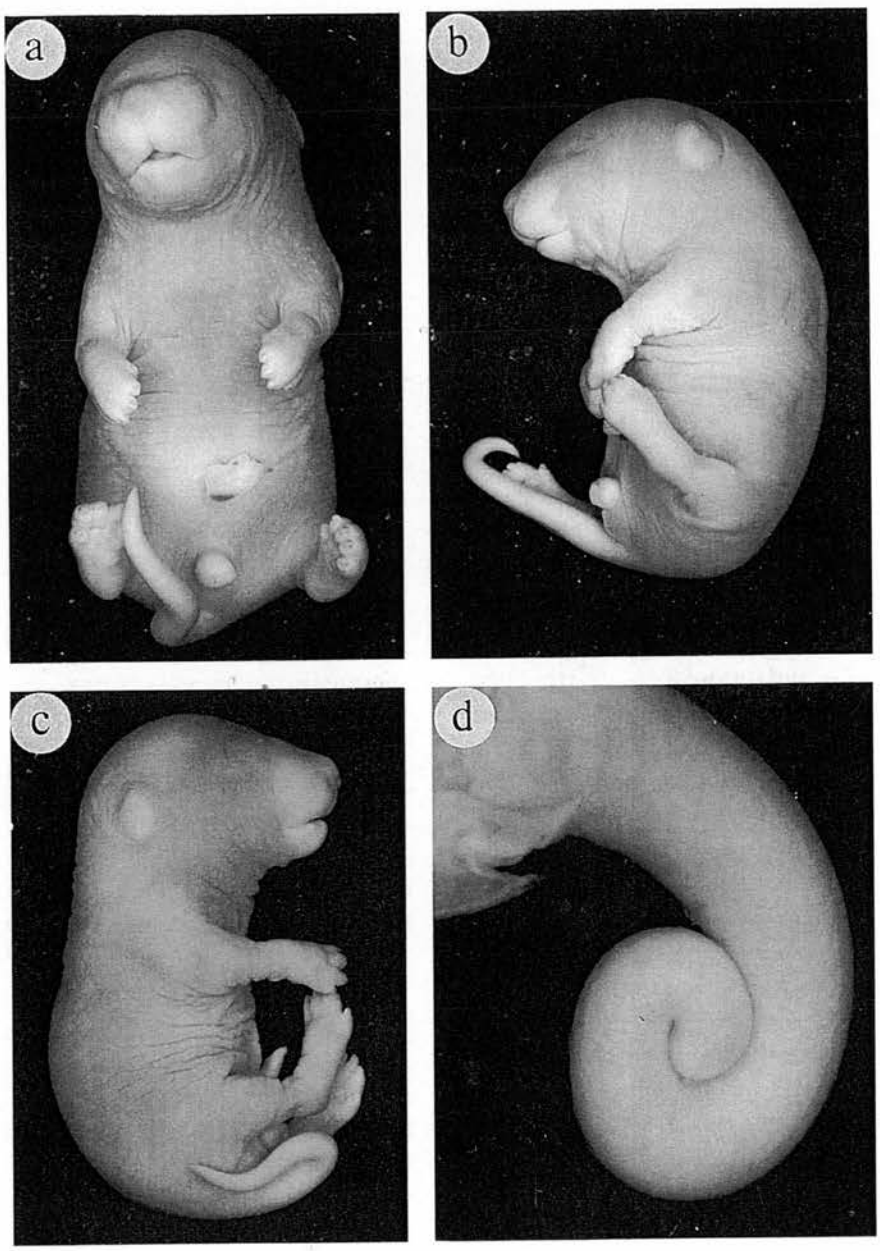
(a) The appearance of a "kinked" tail. A Frontal view of the affected fetus.

(b) A tail with a distal "hook". A lateral view of the affected fetus. Note also the abnormal posture of the the left hindlimb.

(c) A tail showing a "double-kinked; a lateral aspect

(d) Lateral view of tail showing an unusual spiral.

Approximate magnification: a-c, x4; d, x 10


a
b
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d

Chapter 3: Limb and other postcranial skeletal defects induced by amniotic sac puncture in the mouse

3.1 Introduction

In chapter 2, a mouse model for studying the teratogenic effects of amniotic sac puncture has been described and the possible mechanisms involved have been discussed. The abnormalities induced by amniotic sac puncture in the mouse included cleft palate, limb and tail deformities.

Similar teratogenic effects of amniotic sac puncture have been reported in the rat (Poswillo, 1966; DeMyer and Baird, 1969; Love and Vickers, 1972; Singh and Singh, 1973; Singh *et al.*, 1974; Kino, 1975; Kennedy and Persaud, 1977; Houben, 1980, 1984; Houben and Huygens, 1987). Histological examination of the rat fetuses indicated that the abnormal pattern of chondrification, and subsequent differentiation of the skeletal rudiments might have been caused by vascular disturbance (Love and Vickers, 1972; Houben, 1984). Furthermore, some rat studies revealed longitudinal splitting (dichotomy) of the distal part of the phalangeal elements of the digits and acrosyndactyly (fusion of the terminal portion of two or more digits, with cleft or sinuses present between their proximal phalanges) of the distal phalanges (Love and Vickers, 1972; Kino, 1975). In the mouse, however, no information was available as to whether similar effects might have been induced.

The principal aims of this chapter are therefore to (1) measure the lengths of the intact bone and the lengths of ossification centres in the long bones, and to establish whether there was any selective effect(s) of amniotic sac puncture on chondrification or ossification on the proximal or distal elements of the limb skeleton, (2) evaluate the effects of this procedure on the wrist and ankle joints, and to establish whether the degree of severity observed differed between the forelimbs and the hindlimbs and between the two sides, (3) examine in detail the skeletal elements of the 'hands' and 'feet', (4) compare the soft tissue anomalies with those of the underlying skeleton, and (5) provide observations on the unexpected appearance of the sternum in a proportion of the 'experimental' fetuses.

In order to investigate the effect of amniotic sac puncture on the mouse postcranial skeleton, mouse embryos previously exposed to amniotic sac puncture on d 13 were analysed on d 19 of pregnancy. The embryos were subjected to a "clearing" and double-stain method using alizarin red S and Alcian blue to reveal respectively their bony and cartilaginous elements.

3.2 Materials and Methods

3.2.1 Mouse (details see section 2.2.1)

3.2.2 1 Preparation of Avertin and anaesthesia (details see section 2.2.2.1)

3.2.2.2 Midline laparotomy (details see section 2.2.2.2)

3.2.2.3 Amniotic sac puncture and post-puncture procedures (details see section 2.2.2.3)

3.2.2.4 Dissection on day 19.0 (details see section 2.2.2.4)

3.2.3 Production of "internal", and "non-experimental" control and "experimental" groups

At about 10.00 a.m. on day 19 of pregnancy, all females were sacrificed by cervical dislocation. The uterine horns were removed and the fetuses were isolated and separated into "internal" control and "experimental" groups according to the operating records. In addition, a "non-experimental" control group was also produced, namely fetuses isolated from pregnant females on day 19 of gestation that had not previously been anaesthetised or exposed to a laparotomy.

3.2.4 Alcian blue and alizarin red S stain for skeletal elements

The fetuses from the 'internal' control and those in the "experimental" groups were killed by chloroform inhalation and fixed in 80 % ethanol at room temperature for one week. The skin was removed and fetuses were dehydrated in 96 % ethanol for 12 - 24 hours followed by acetone for 3 days according to the method of C. Arnott (Kaufman, 1992). The specimens were then bulk stained with alizarin red S and Alcian blue. The material was then cleared with potassium hydroxide and stored in glycerine using a modification of the technique described by Meyer and O'Rahilly (1958, see Kaufman, 1992). The forelimbs and hindlimbs were disarticulated from the axial skeleton at the sternoclavicular and sacroiliac joints, respectively. The skeletal elements from an additional ("non-experimental") control group were also analysed.

3.2.5 Measurements of skeletal elements

Various measurements were made on the skeletal elements of fetuses from these three groups, namely a. "experimental" fetuses, b. "internal" controls, and c. "non-experimental" controls, each group being as defined above. The intact lengths of the cartilage models of the humerus, ulna, femur, tibia, the middle metacarpal and metatarsal bones were all measured using a graticule eyepiece under the x 6 or x 12 magnification of a Wild M5 stereomicroscope (Patton and Kaufman, 1995). Each measurement was made on three separate occasions. The average calculated from these three values was used in subsequent analyses. In order to obtain consistent results, all the specimens were analysed under the following

uniform conditions: (1) the specimen were put in identical dishes which contained the same depth of glycerine, (2) the specimens faced the same direction, and (3) the specimen were retained in the middle of the field of view. The ossification lengths of the long bones were also obtained in a similar way, though those of the middle metacarpal and metatarsal bones were too small to be measured accurately by this means. Both the intact bone lengths (i.e. the lengths of the cartilage models) and the ossification lengths were converted into mm, and these values are presented in the various tables.

3.2.6 Analyses of variance

Data of both intact bone length and ossification length were subjected to analyses of variance. If the interaction terms were significant, further *t* tests were performed. In about 5 % of cases, where data was not available (usually due to unacceptable mechanical damage during the clearing procedure), the missing values were estimated using a least squares technique and were then substituted into the raw data.

In order to test the hypotheses that the distal values (ulna and tibia) might have been more affected than the proximal ones (humerus and femur) in the experimental group, ratios of each bone length between the "internal" control group and the "experimental" group were also examined. It was more convenient to perform a log transformation of the data, since a ratio is the difference between logs. For both intact bone length and ossification length the log transformation was satisfactorily achieved. Before analysing the data, histogram plots of residuals were made together with normal probability plots to confirm the normality of the data.

3.2.7 Severity scoring of the "clubhand" and "clubfoot"

The degree of severity of the "clubhand" and "clubfoot" (principally talipes equinovarus) deformities was scored based on a zero- to four-point scale. Those with no obvious deformity were scored 0, whilst those with abnormalities of the maximum degree of severity were scored 4. Intermediate degrees of severity were scored accordingly.

In relation to the assessment of "clubhand", in those with a maximum degree of severity, the metacarpal and phalangeal bones were generally angled at about 90° to the ulna and radius, while this angle was closer to 30° in those given a score of 2, and 60° in those given a score of 3. Those instances where there appeared to be a small degree of angulation (5 - 10°) were given a score of 1. A similar system was used in assessing the severity of "clubfoot". In the most severe cases, the metatarsals and phalanges were maximally medially rotated and displaced by about 90° to the normal situation. These were given a score of 4. Intermediate degrees of medial rotation were scored accordingly, so that those with about 30° of

medial rotation were given a score of 2, while those with nearer to 60° of medial rotation were given a score of 3.

The degree of severity of the deformities were determined independently by two observers, and in those few instances where a difference of more than one point was obtained, the region concerned was rescored and a consensus value agreed.

The morphology of each component bone was investigated, with a particular interest in the phalanges and the bones of the wrist and ankle regions. All limbs were examined in order to establish the incidence of anomalies.

3.2.8 Viewing the ossification centres in the sternum

In the sternum, the pattern of ossification in the 'experimental' group was so variable that a drawing was made for each specimen, and the more unusual patterns of ossification were photographed. In order to view more clearly the ossification centres in the sternum, and to facilitate the photography of this region, the ventral half of the chest wall was removed as an intact unit by sectioning through the clavicles and the rib cage just behind the costo-chondral junctions

3.3 Results

3.3.1 General observations

A total of 27 pregnant females were anaesthetised on day 13 of pregnancy. The left uterine horn was exteriorized in 15 females, and the right horn in 12 females, and amniotic sac puncture performed on the exteriorized sides. In the "internal" control series, a total of 104 living fetuses with normal morphology were recovered, and a total of 7 resorptions were noted.

In the "experimental" series, no statistical difference was observed in the incidence of morphologically normal or abnormal living fetuses or resorptions observed in the left or right uterine horns, and accordingly the results from the two sides were pooled. The pooled data are as follows: total morphologically normal living fetuses, 28; total living fetuses with external morphological abnormalities, 40; total resorptions, 68.

Nine fetuses from the "non-experimental" control series were examined in detail in order to obtain baseline measurements of intact bone lengths and ossification lengths of the various long bones of the

limbs studied. Ten well-stained fetuses were selected at random from the "internal" control series and various measurements taken as indicated in the material and methods. As the statistical analysis on this material indicated that in all respects it was not significantly different from the "non-experimental" controls, no additional fetuses from this series were analysed.

The mean crown-rump lengths (±sem) of the various groups studied are as follows: "internal" control fetuses 22.36 ± 0.11 mm (n= 104); morphologically normal "experimental" fetuses 21.82 ± 0.32 mm (n= 28); morphologically abnormal "experimental" fetuses 19.09 ± 0.34 mm (n= 40).

As the mean crown-rump length of the morphologically normal fetuses from the "experimental" series was not significantly different from that of the "internal" control fetuses, it was assumed that the former group had probably not been subjected to the teratogenic effect of amniotic sac puncture - possibly because only a limited rather than an extensive loss of amniotic fluid had occurred in these cases. Because the aim of this study was to investigate the teratogenic effect of amniotic sac puncture on the post-cranial skeleton, with a particular interest in its effect on inducing limb abnormalities, these fetuses were not examined further. Accordingly, only those fetuses from the "experimental" series that *displayed external morphological abnormalities* were subjected to further detailed analysis. Out of a total of 40 fetuses in this category, 38 were well-stained and the limbs were either undamaged or had minimal mechanical damage at the end of the double-staining and clearing procedure, and these were analysed in detail (see below). The term "experimental" fetuses therefore refers, from this point of the present study, exclusively to the subgroup from that series that displayed external morphological abnormalities.

3.3.1.1 Intact bone length

The mean values of intact bone lengths, as determined by measuring the length of the cartilage primordia of the humerus, ulna, femur and tibia, and the middle metacarpal and metatarsal bones of fetuses from the "internal" control, "non-experimental" control and "experimental" groups are provided in Table 3.1, and the results of analyses of variance in logs are provided in Table 3.2. Accordingly, this analysis confirms that there was no significant difference between the two sides with respect to intact bone length in the two control groups and in the experimental group. However, the experimental values were invariably smaller (average 90.62 % for the humerus, ulna, femur and tibia; 94.56 % for metacarpal and metatarsal) than in both control series. Equally, there was no increased effect on the distal compared to the proximal bones of the limbs.

3.3.1.2 Ossification length

The length of the primary centre of ossification in each of the long bones of the forelimb and hindlimb are presented in Table 3.3, and the analyses of variance in log form are also included (see Table 3.4). All interaction effects are significant, therefore further *t* tests were processed, and the results (data not shown) paralleled those seen in the intact bone length studies. No significant difference was found between the right and left side in all series. The length of the ossification centres in the experimental group was reduced by an average of 88.69 % when compared to the control groups. It was observed that the amniotic sac puncture procedure had the same effect on the length of the primary ossification centres of the distal and proximal limb bones.

3.3.2 Effect of amniotic sac puncture on the wrist and ankle joints

3.3.2.1 The wrist joint ("clubhand")

Once the soft tissues had been "cleared" and the skeletal elements double-stained with alizarin red S and Alcian blue, evidence of a "clubhand" deformity was readily recognized. In these cases, a moderate to severe degree of flexion at the carpo-metacarpal joint was apparent (manus flexa), often, though not invariably, associated with ulnar deviation (manus flexa valga), and occasionally associated with radial deviation (manus flexa vara).

The severity of "clubhand" in relation to the left and right forelimbs was scored separately, and the findings are presented in Table 3.5. An analysis of these findings reveals that in both the "internal" and "non-experimental" control series all forelimbs were invariably scored as either 0 or 1. In the "experimental" series, however, the majority of the forelimbs analysed scored between 1 - 4, though the majority scored either 2, 3 or 4, with the left forelimb being the more severely affected, this being apparent from the "severity index" values obtained from this analysis, in which the left forelimbs scored on average 2.38 (n=37), while the right forelimbs scored 1.32 (n=37). 78.3 % of the left forelimbs scored either 2, 3 or 4, while only 43.2 % of the right forelimbs scored within this range, though none in this group received a severity score of 4.

When a comparison is made between the degree of severity of "clubhand" between the two sides, in 67.6 % of fetuses the left forelimb was more severely affected than the right forelimb. In only 18.9 % was the degree of severity similar between the two sides, while in only 13.5 % was the left forelimb found to be

less severely affected than the right forelimb. Typical skeletal abnormalities associated with the forelimb are illustrated in Figure 3.1.

Ulnar deviation was also commonly observed, and seemed largely to be associated with the joint between the ulna and radius and the proximal row of carpal bones. This was apparent in 32.4 % of the left wrists, and in only 8.1 % of the right wrists studied. In the remaining examples, minor damage sustained during the "clearing" procedure precluded this analysis being successfully performed. It was not possible to determine with any degree of accuracy the extent, or degree of severity, of the ulnar deviation present in these cases. It was also noted that ulnar deviation was not necessarily associated with the presence of a "clubhand", though the two conditions were commonly present in the same forelimb.

3.3.2.2 The ankle joint ("clubfoot")

3.3.2.2.1 Incidence of "clubfoot" deformity

While the degree of severity of "clubfoot" in all of the left and right hindlimbs in both the "internal" and "non-experimental" control groups scored within the range 0-1, those in the "experimental" series mostly scored within the range 1 - 4. In the left hindlimb 76.7 %, and in the right hindlimb 85.3 % scored within this range, with 53.3 % and 41.2 % of the left and right hindlimbs, respectively, scoring either 2, 3 or 4 (see Table 3.6).

However, no statistically significant difference was found in the degree of severity of "clubfoot" encountered between the left and right sides. Out of a total of 28 fetuses in which it was technically possible to undertake such a comparison, where both hindlimbs were intact and undamaged following the "clearing" procedure, in 32.1 % the left side was more severely affected than the right side, in 39.3 % the two sides were equally severely affected, and in the remaining 28.6 %, the left side was less severely affected than the right side. Typical skeletal abnormalities associated with the hindlimb are illustrated in Figure 3.2.

3.3.2.2.2 Deformity in the shape of the calcaneus

In a small proportion of cases, which were all confined to the "experimental" series, the plantar aspect of the calcaneus had an exaggerated arch-like shape (see Figure 3.2). This involved the region between the calcaneal tuberosity and the anterior tubercle, and was in marked contrast to the relatively flattened or minimally concave appearance of this region in the control fetuses and in the majority of the fetuses in the "experimental" series. Lesser degrees of concavity of this region, which were also undoubtedly

morphologically abnormal, were also observed in the "experimental" series, but were found to be too difficult to quantify.

3.3.2.3 Effects on the phalanges

The total number of skeletal abnormalities involving the phalanges encountered in this study was comparatively small, and principally involved the distal phalanges of the middle three digits. The proximal and middle rows of phalanges in the "experimental" series tended to be somewhat shorter and more cylindrical in shape than were the comparable phalanges in the control series, and the distal phalanges tended to be more squat than those in the control series. Where particularly gross skeletal abnormalities of the phalanges were encountered, they were almost invariably associated with one or more of the distal phalanges. A selection of the phalangeal abnormalities encountered in this series are illustrated in Figures 3.1 and 3.2. The incidence of "clubhand" and "clubfoot" deformities and other major skeletal abnormalities of the hand and foot regions are summarized in Table 3.7.

3.3.2.4 Intervening skeletal element acrossing distal phalanges

Occasionally, an intervening skeletal element was observed "bridging" neighbouring phalanges in the distal parts (Figure 3.2 d, e).

3.3.3 Morphological appearance of the sternum and rib cage

In all of the control fetuses, the sternum was relatively narrow and formed by a series of midline square-shaped cartilaginous units (the sternebrae) between which the first seven costal cartilages are inserted. By day 19, a single discrete centre of ossification is usually seen in association with each of the sternebrae. The manubrium and xiphoid process each usually possess a single large centre of ossification, though the xiphoid process may contain two centres of ossification, one on either side of the midline.

3.3.3.1 The sternum

The most curious finding in this component of the study concerns the anomalous pattern of ossification of the sternum seen in about two-thirds of the fetuses in the "experimental" series. In 13 out of a total of 38 fetuses examined in the "experimental" series, the appearance of the ossification centres was either completely normal or very similar to the normal pattern. In the remaining 25 cases, the pattern of ossification seen in relation to the entire sternum gave it, in 2 instances, either an irregular "chequered" or "crankshaft" appearance, or (in the remaining 23 cases) a more bizarre pattern, often involving fusion

between two or more adjacent sternebral ossification centres. Occasionally, fusion was present between all of the sternebral ossification centres. The pattern of ossification varied from one fetus to another with no obvious underlying pattern. A selection of the various anomalous patterns of ossification seen in relation to the sternum are illustrated in Figure 3.3.

3.3.3.2 The rib cage

In 50 % (19 out of 38) of the fetuses in the "experimental" series, the rib cage had an asymmetrical appearance. In the majority of these cases, the 5th to 7th costal cartilages and their associated ribs were displaced unevenly and cranially. In 17 of these 19 fetuses, it was the left side of the rib cage that was particularly distorted and compressed.

3.4 Discussion

3.4.1 Shortening of skeletal elements

The influence of amniotic sac puncture on the skeletal elements of the limbs and thoracic cage has been investigated in the present study. The intact cartilaginous primordia of the proximal and distal long bones of the forelimb and hindlimb have been examined. The results revealed that the shortening of these bones seen in the "experimental" fetuses with external morphological malformations might be accounted for by the overall reduction in the crown-rump length of these fetuses. However, the greater than expected decrease in the lengths of the primary centres of ossification seen in this subgroup of the "experimental" series, when compared to those of the controls, suggested that this phenomenon might have been accounted for by a greater than expected degree of developmental delay, or temporary arrest, induced by the amniotic sac puncture.

3.4.2 Low incidence of skeletal abnormalities

The incidence of gross skeletal abnormalities encountered in this study was surprisingly low, which was similar to those reported by others (Love and Vickers, 1972; Kino, 1972), apart from those associated with the ossification of the sternum, a finding which has not previously been reported (see below). While the incidence of soft tissue abnormalities in this model was particularly high, this was clearly not reflected in transverse amputations involving the distal skeletal elements of the limbs.

3.4.3 Abnormalities in the phalanges

Distal phalangeal anomalies, such as fusion between the phalanges of adjacent digits, were occasionally encountered, although no examples were seen in which the phalanges were absent. Phalanges were seen even in cases of adactyly, where no obvious digits were present at the soft tisue level. In the human, cases of symphalangism have been occasionally reported in infants exposed to CVS during early pregnancy (Report of NICHHD Workshop, 1993). Following a similar experimental approach in the rat, the presence of dichotomy involving the metacarpal/metatarsal and phalangeal skeletal elements has been reported in the majority of specimens (Love and Vickers, 1972; Kino, 1972; Kennedy and Persaud, 1977). We have only observed this phenomenon on five occasions. Our findings, however, appear to more closely resemble the phalangeal abnormalities seen clinically by Kino (1972).

3.4.4 "clubfoot" and "clubhand" deformities

While we had previously observed a relatively high incidence of "clubfoot" deformity in fetuses previously exposed to amniotic sac puncture (MacIntyre *et al.,* 1995), the availability of "cleared" specimens has allowed the degree of severity of this condition to be established with a greater degree of accuracy. In addition, this approach has revealed that similar deformities are also induced in relation to the wrist joint, and that the degree of severity may also be determined with some degree of accuracy.

It has been suggested that a postural or mechanical component is involved in a proportion of children with "clubfoot" (Duthie and Bentley, 1983). For example, "clubfoot" has been associated with a persistent complete breech presentation. According to Duthie and Bentley (1983), "*environmental factors such as intrauterine compression through change in the size of the uterus or reduction in the amount of amniotic fluid has been described over many years, but surprisingly little is known about intrauterine biomechanics to substantiate such observations*". It is also postulated that these factors probably act during the period between 30 weeks of gestation and term. This group, which appears to have a postural basis, tends to respond well to conservative management.

In at least some cases of "clubfoot", however, there appears to be an abnormality in the shape of the talus/calcaneus which may be genetically determined (Wyne-Davis, 1964a, 1964b; Palmer, 1964), and these tend to be resistant to correction even by operation. In the amniotic sac puncture experiments, both the "clubfoot" and "clubhand" deformities seen must be postural rather than genetic in origin, as neither of these conditions are seen in the control series.

One of the questions that immediately comes to mind from these studies is why should there be a higher incidence of hindlimb compared to forelimb abnormalities? The most likely explanation for this observation is that the forelimbs are about 12 hours more advanced in development than the hindlimbs when amniotic sac puncture is carried out, and that the less well developed structures are likely to be more sensitive to teratogenic stimuli.

In the study in chapter 2, virtually all of the forelimb abnormalities included in this analysis involved the soft tissues of the digits. It was also shown in chapter 2 that about 80 % of the hindlimb abnormalities observed related to the digits, and 20 % to the presence of a "clubfoot" deformity. In the present study, the "clearing" of the soft tissues has revealed an underlying incidence of ankle and wrist abnormalities that were not formerly recognised; 78.3 % of the left forelimbs showed evidence of some degree of "clubhand", compared to 43.2 % of the right forelimbs, the difference between the two sides being significant. In relation to the ankle region, 53.3 % and 41.2 % respectively of the left and right hindlimbs showed evidence of some degree of "clubfoot", there being no significant difference between the incidence on the two sides.

A postural difference may be the simplest explanation for the difference observed between the incidence of "clubhand" between the two forelimbs. During days 12 - 13, the tail is more often directed towards the right than to the left of the head, with the head being directed slightly towards the left side of the midline. Following amniotic sac puncture, the resultant oligohydramnios probably causes compression of the head onto the thorax. However, since the head is already directed slightly towards the left, the resultant compression induced by the oligohydramnios may accentuate this asymmetry, so that the left forelimb may be subjected to a greater degree of compression by the mandibular region than the right forelimb, with the uterine wall and extra-embryonic membranes acting as a constraining influence.

This accentuated movement of the mandibular region towards the left side following oligohydramnios may also provide a partial explanation for the asymmetry of the thoracic wall abnormalities seen in the "experimental" series, where in 17 of 19 affected fetuses, it was the left side of the rib cage that was the most distorted and compressed. Love and Vickers (1972) carried out similar experiments in the rats and also noted that the left side was more severely affected than the right in relation to both the forelimb and hindlimb. While the explanation proposed above may account for the forelimb abnormalities seen by these authors, the reason for the asymmetric effect on the hindlimb has yet to be determined. It should be noted, however, that in our study neither intact bone lengths nor lengths of the ossification centres of the long bones of the two sides were asymmetrically affected.

3.4.5 Abnormalities in the sternum

The unexpected anomalous pattern of ossification of the sternum observed in a high proportion of the "experimental" fetuses that displayed external morphological abnormalities but not in the control series of fetuses nor in the fetuses in the "experimental" series that displayed no external morphological abnormalities has, as far as we are aware, only been reported previously in relation to *En-1* $^{hd/hd}$ homozygous mutant mice (Wurst *et al.* 1994). While it is likely that this phenomenon, as with the limb and other anomalies reported here and previously, may have a postural basis, it is possible that the change in amniotic sac pressure following puncture may interfere either directly or indirectly with the normal expression pattern of certain critical pattern forming genes, such as *Engrailed-1* that control the ossification of the sternal elements. If this is the case, then some of the limb and sternal malformations observed in relation to our model system, may result from a combination of mechanical deformity and/or interference with normal pattern forming gene activity.

3.4.6 Clinical relevance of the present study

The findings of the present study may be clinically relevant, in that they shed light on why about 2 % of infants exposed to chorionic villus sampling carried out during the first trimester of pregnancy (but particularly during days 55 - 65 of gestation) display evidence of craniofacial and/or limb abnormalities, a condition termed the oromandibular-limb hypogenesis syndrome (Planteydt *et al.,* 1986; Christiaens *et al.*, 1989; Kaplan *et al.*, 1990; Editorial, 1991; Firth *et al.*, 1991; Hsieh *et al.*, 1991; Mahoney, 1991; Mastroiacovo and Cavalcanti, 1991; Rodriguez and Palacios, 1991; Burton *et al.*, 1992; Froster and Baird, 1992; Kuliev *et al.,* 1992; Schloo *et al.,* 1992). While the relationship between CVS and the birth of infants with this syndrome has yet to be established (for recent discussion, see van Allen, 1992; Winter, 1994), it appears likely that in at least a finite number of cases, the inadvertent puncturing of the amniotic sac must occur during this procedure. This may result in the leakage of a significant volume of amniotic fluid, and induce a degree of oligohydramnios (Kaufman, 1994). We believe that a model system that simulates this event could provide clues which might help to explain the pathogenesis of the limb (and craniofacial) malformations seen in these infants.

	'Nonexperimental' controls	'Internal' controls	Experimentals‡
Humerus			
Left	4.60±0.027* (8)†	4.57±0.043 (10)	4.20±0.057 (36)
Right	4.65±0.034 (9)	4.61±0.052 (10)	4.23±0.054 (36)
Ulna			
Left	4.72±0.020 (6)	4.64±0.054 (9)	4.29±0.053 (34)
Right	4.73±0.035 (7)	4.66±0.047 (10)	4.29±0.063 (34)
Metacarpal			
Left	1.12±0.019 (6)	1.14±0.013 (9)	1.05±0.016 (34)
Right	1.15±0.013 (7)	1.15±0.015 (10)	1.06±0.013 (33)
Femur			
Left	3.90±0.033 (9)	3.85±0.050 (10)	3.48±0.059 (37)
Right	3.96±0.035 (8)	3.85±0.049 (10)	3.52±0.067 (35)
Tibia			
Left	4.18±0.038 (8)	4.14±0.049 (9)	3.83±0.052 (37)
Right	4.16±0.037 (6)	4.14±0.056 (8)	3.79±0.060 (33)
Metatarsal			
Left	1.58±0.020 (8)	1.57±0.015 (9)	1.43±0.021 (34)
Right	1.55±0.018 (6)	1.54±0.011 (9)	1.44±0.018 (31)

* Mean ± S.E.M.; unit: mm; † number in parentheses refers to the sample size; ‡ only those with external abnormalities analysed (see text).

Table 3.1 Determination of intact bone length. The mean values of intact bone lengths, as determined by measuring the length of the cartilage primordia of the humerus, ulna, femur and tibia, and the the middle metacarpal and metatarsal bones of fetuses from the "internal" control, "non-experimental" control and "experimental" groups are listed. All measurements were made using a graticule eyepiece under the x 6 or x 12 magnification of a Wild M5 stereomicroscope as described by Patton and Kaufman (1995). Each measurement was made on 3 separate occasions, and the average calculated from these 3 values was used. In order to obtain consistent results, all the specimen were analysed in identical dishes which contained the same depth of glycerine, the specimen faced the same direction, and were retained in the middle of the field of view. The measurements on graticule units were converted into mm and listed in the table.

Source of variation	D.F.	Sum of squares	Mean square	F ratio	Probability (P)
Treatment	2	1.345	0.672	10.89	< 0.001
Error	53	3.273	0.0617		
Bone	5	2118	42.73	34800	< 0.001
Side	1	0.00221	0.00221	1.81	0.179
Bone × treatment	10	0.0172	0.0172	1.41	0.171
Bone × side	5	0.0103	0.00205	1.68	0.137
Treatment × side	2	0.000718	0.000359	0.29	0.745
Bone × treatment × side	10	0.00541	0.000541	0.44	0.924
Error	526	0.641	0.00122	—	—
Total	614	1942	—	—	—

Treatment: 'nonexperimental' control, 'internal' control, 'experimental'; side: left, right.

Table 3.2 Analysis of variance: log intact bone length. The data is transformed into a log form for the convenience of comparing the difference between ratios. Before analysing the data, histogram plots of residuals were made together with normal probability plots to confirm the normality of the data. This analysis confirms that there was no significant difference between the 2 sides with respect to intact bone length in the two control groups and in the experimental group. However, the experimental values were invariably smaller for all bones studied than comparable bones in the "internal" control series. Equally, this analysis also confirms that there was no increased effect on the distal compared to the proximal bones of the limb.

	'Nonexperimental' controls	'Internal' controls	Experimentals‡
Humerus			
Left	2.83 ± 0.026* (8)†	2.91 ± 0.037 (10)	2.58 ± 0.052 (36)
Right	2.81 ± 0.037 (9)	2.92 ± 0.043 (10)	2.62 ± 0.054 (36)
Ulna			
Left	3.11 ± 0.037 (6)	3.21 ± 0.053 (9)	2.85 ± 0.055 (34)
Right	3.09 ± 0.034 (9)	3.15 ± 0.055 (10)	2.81 ± 0.060 (35)
Femur			
Left	2.25 ± 0.027 (9)	2.32 ± 0.052 (10)	2.06 ± 0.047 (37)
Right	2.27 ± 0.029 (8)	2.36 ± 0.039 (10)	2.10 ± 0.049 (35)
Tibia			
Left	2.66 ± 0.049 (8)	2.84 ± 0.096 (10)	2.54 ± 0.062 (37)
Right	2.64 ± 0.059 (7)	2.85 ± 0.077 (8)	2.53 ± 0.066 (34)

* Mean ± S.E.M.; unit: mm; † the numbers in parentheses refer to the sample size; ‡ only those with external morphological abnormalities analysed (see text).

Table 3.3 Determination of ossification length. The length of the primary centre of ossification in each of the long bones of the forelimb and hindlimb are listed. The method of measurement was the same as described in the legend of table 3.1.

Source of variation	D.F.	Sum of squares	Mean square	F ratio	Probability (P)
Treatment	2	1.9458	0.9729	7.66	0.001
Error	53	6.7287	0.1270		
Bone	3	6.2720	2.0907	1750	< 0.001
Side	1	0.0650	0.0650	54.42	< 0.001
Bone × treatment	6	0.7208	0.1201	100.62	< 0.001
Bone × side	3	0.1528	0.0509	42.66	< 0.001
Treatment × side	2	0.2120	0.1060	88.67	< 0.001
Bone × treatment × side	6	0.7038	0.1173	98.24	< 0.001
Error	348	0.4155	0.001194		
Total	424	16.3935			

Treatment: 'nonexperimental' control, 'internal' control, 'experimental'; side: left, right.

Table 3.4 Analysis of variance: log ossification length. The data is transformed into a log form for the convenience of comparing the difference between ratios. Before analysing the data, histogram plots of residuals were made together with normal probability plots to confirm the normality of the data. This analysis shows no significant difference between the right and left sides in all series. It also shows that the amniotic sac puncture procedure had the same effect on the length of the primary ossification centres of the distal and proximal limb bones.

	Left hand			Right hand		
Severity*	'Nonexperimental' controls	'Internal' controls	Experimentals	'Nonexperimental' controls	'Internal' controls	Experimentals
Degree 0	7 (87.5%)	9 (90.0%)	1 (2.7%)	6 (66.7%)	8 (80.0%)	7 (18.9%)
Degree 1	1 (12.5%)	1 (10.0%)	7 (18.9%)	3 (33.3%)	2 (20.0%)	14 (37.8%)
Degree 2	0	0	13 (35.1%)	0	0	13 (35.1%)
Degree 3	0	0	9 (24.3%)	0	0	3 (8.1%)
Degree 4	0	0	7 (18.9%)	0	0	0
Total	8 (100.0%)	10 (100.0%)	37 (100.0%)	9 (100.0%)	10 (100.0%)	37 (100.0%)

*Those with no obvious deformity were scored 0, while those with abnormalities of the maximum degree of severity were scored 4. Intermediate degrees of severity were scored accordingly.

Table 3.5 The severity of "clubhand" observed in the experimental, "non-experimental", and "internal" control groups. Evidence of "clubhand" deformity was readily recognized after the soft tissues had been "cleared" and the skeletal elements double-stained with alizarin red S and Alcian blue. Note that all forelimbs invariably scored as either 0 or 1 in both the "internal" and "non-experimental" control series. This is in contrast to the majority of the forelimbs that scored between 1 - 4. This table also shows that the left forelimb were less severely affected than the right forelimb.

	Left foot			Right foot		
Severity*	'Nonexperimental' controls	'Internal' controls	Experimentals	'Nonexperimental' controls	'Internal' controls	Experimentals
Degree 0	4 (50.0%)	7 (77.8%)	7 (23.3%)	5 (62.5%)	5 (55.6%)	5 (14.7%)
Degree 1	4 (50.0%)	2 (22.2%)	7 (23.3%)	3 (37.5%)	4 (44.4%)	15 (44.1%)
Degree 2	0	0	6 (20.0%)	0	0	5 (14.7%)
Degree 3	0	0	9 (30.0%)	0	0	7 (20.6%)
Degree 4	0	0	1 (3.3%)	0	0	2 (5.9%)
Total	8 (100.0%)	9 (100.0%)	30 (100.0%)	8 (100.0%)	9 (100.0%)	34 (100.0%)

*Those with no obvious deformity were scored 0, while those with abnormalities of the maximum degree of severity were scored 4. Intermediate degrees of severity were scored accordingly.

Table 3.6 The severity of "clubfoot" observed in the experimental, "non-experimental", and "internal" control groups. Evidence of "clubfoot" deformity, like that of "clubhand", was readily recognized after the soft tissues had been "cleared" and the skeletal elements double-stained with alizarin red S and Alcian blue. Note that while the degree of severity of "clubfoot" in all of the left and right hindlimbs in both the "internal" and "non-experimental" control groups scored within the range of 0 - 1, the majority in the "experimental" series scored within the range of 1 - 4. There was, however, no statistically significant difference in the degree of severity of "clubfoot" encountered between the left and right sides.

	'Clubhand' (score 2–4)	Ulnar deviation	'Clubfoot' (score 2–4)	Abnormally concave calcaneus	Phalangeal abnormalities		
					Diastodactyly	Acrosyndactyly	Duplication (forked)
LFL (n = 37)	29	12	N.A.	N.A.	7	1	3
RFL (n = 37)	16	3	N.A.	N.A.	1	1	1
LHL (n = 40)	N.A.	N.A.	16	5	3	7	1
RHL (n = 34)	N.A.	N.A.	14	3	1	3	0

Table 3.7 Summary of the incidence of "clubhand" and "clubfoot" deformities and other major skeletal abnormalities found in the hand and foot regions.

Figure 3.1 Ossification of the bones of the distal part of the forearm "cleared" and stained with alizarin red S (to display ossification centres) and Alcian blue (for cartilage). A specimen from a control fetus is illustrated in (a) and specimens from "experimental" fetuses previously exposed to amniotic sac puncture on day 13 are illustrated in (b) to (f). The developmental stage given for each specimen is based on the detailed staging system as described by Patton and Kaufman (1995), which is based on the number and appearance of the ossification centres present.

(a). Dorsal view of the right forelimb of a control fetus isolated on day 19. Ossification centres are present in the ulna, radius, metacarpals 2 - 5 (the 1st metacarpal is obscured, but has no centre of ossification) and in the proximal and distal rows of phalanges. Stage equivalent to day 19 to day 19+.

(b). Dorsal view of the right forelimb of an "experimental" fetus. Centres are present in the 2nd to 5th metacarpals, the 3rd and 4th proximal and 2nd, 3rd and 4th distal phalanges. The hand region appears flattened and compressed longitudinally. The interphalangeal joints are only poorly developed. Stage equivalent to day 18+.

(c). Severe degree of radial "clubhand", with abnormal pattern of ossification. An ossification centre appears to be present in the 1st metacarpal, although this is not usually seen before day 1 and about 1 week after birth. Centres are also present in the 1st and 5th proximal and in what appears to be an anomalous 5th distal phalanx, and in the 2nd and 3rd (fused) and 4th distal phalanges. The 2nd and 3rd middle phalanges appear to be fused together and share a distal single phalanx. Stage equivalent to day 18+.

(d). Severe degree of radial "clubhand". Fusion is present between the distal phalanges of digits 3 and 4. Ossification centres are present in the proximal part of the 5th and distal part of the 2nd digits, though not in the equivalent regions of the 3rd and 4th digits where centres would be expected to be present. Stage equivalent to day 18+.

(e). Severe degree of "clubhand". Stage equivalent to day 18+.

(f). Severe degree of "clubhand". Stage equivalent to day 19.

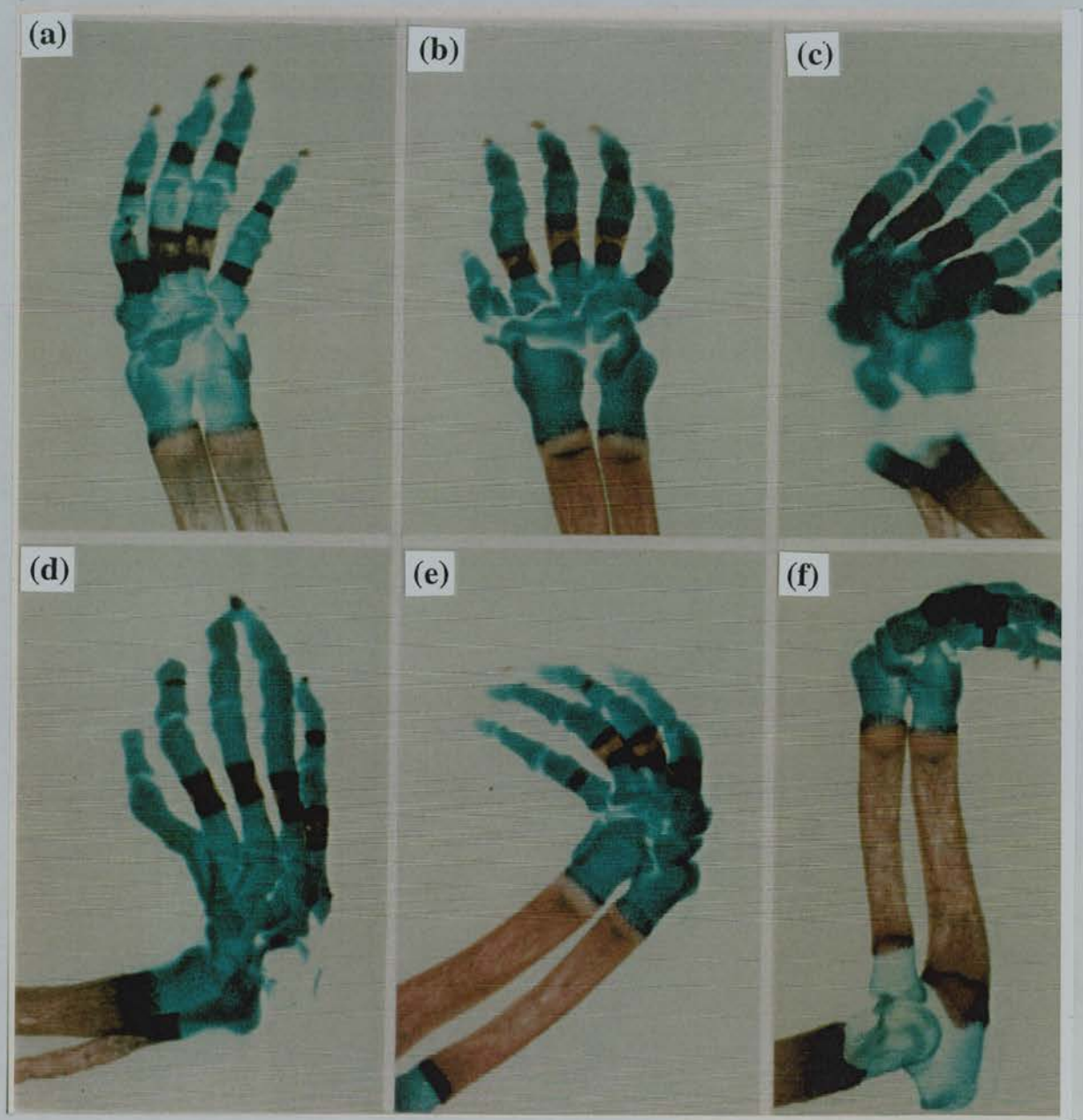

(a)
(b)
(c)
(d)
(e)
(f)

Figure 3.2 Ossification of the bones of the distal part of the hindlimb, demonstrated by the same technique described in the legend of figure 3.1. A specimen from a control fetus is illustrated in (a) and from 'experimental' fetuses in (b) to (f). The staging system used is the same as described in the legend of figure 3.1.

(a). Lateral view of the left hindlimb of a control fetus isolated on day 19. Ossification centres are present in all of the metacarpals and the proximal and distal row of phalanges. A small centre is also present in the talus. Stage equivalent to day 19 to day 19+.

(b). Moderate degree of "clubfoot". Centres are present in the 2nd to 5th metacarpals. The distal phalanges of the 2nd, 3rd and 4th digits appear to be missing, while the middle phalanx of the 3rd digit is enlarged and wedge-shaped. The distal phalanx of the 5th digit is duplicated. Stage equivalent to d 17 to d 18.

(c) Moderate to severe degree of "clubfoot". The distal phalanges of digits 2, 3 and 4 are fused together. Stage equivalent to day 18.

(d) Moderate to severe degree of "clubfoot". The distal phalanges of digits 2,3, and 4 are fused together. Stage equivalent to d 18.

(e) Severe degree of "clubfoot". The distal phalanx of the 3rd digit is grossly abnormal, and fused to the distal element of digit 4. Stage equivalent to d 18.

(f). Moderate to severe degree of "clubfoot". The distal phalanx of the 3rd digit is enlarged and wedge-shaped, and fused to the distal phalanges of digits 2 and 4. Stage equivalent to day 18.

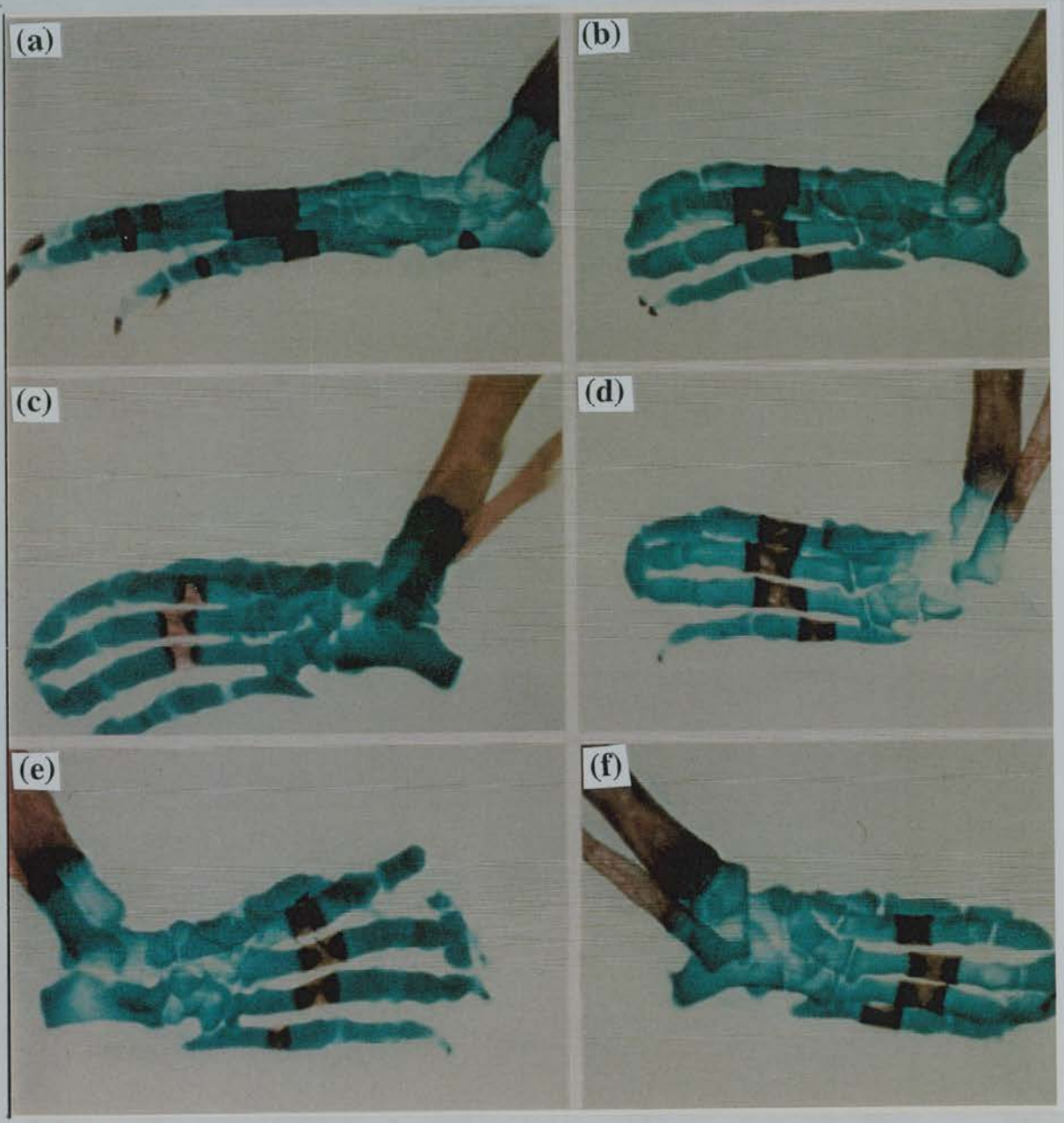

(a)
(b)
(c)
(d)
(e)
(f)

Figure 3.3 Ossification pattern in the sternum of control (in (a)) and "experimental" (in (b) to (l)) embryos exposed to amniotic sac puncture on day 13. All micrographs in this figure are shown at the same magnification (Bar, 2mm).

(a). The normal appearance of the sternal ossification centres on day 19. A single centre is present in the manubrium and in the xiphisternum and in the 4 intervening sternebrae. Note that a similar arrangement is seen 1 day earlier, although the ossification centres are only present in the upper 3 sternebrae, and the xiphisternum often possesses 2 centres that are seen to be united across the midline.

(b). This sternum shows a near-normal pattern of ossification, although the sternebral centres are somewhat oblique.

(c). The upper 3 sternebral centres are present although moderately oblique. The 4th sternebral centre is smaller than normal and 2 substantial but widely separated centres are present in the xiphisternum.

(d). Only 3 sternebral centres are present in association with the 2 distinct xiphisternal centres.

(e). The 2nd and 3rd sternebral centres are fused together and associated with 2 obliquely located lateral and a small midline xiphisternal centre.

(f). Three large sternebral centres are present with minimal evidence of fusion between them. These are associated with 2 distinct xiphisternal centres, the one on the left side being fused to the lower sternebral centre.

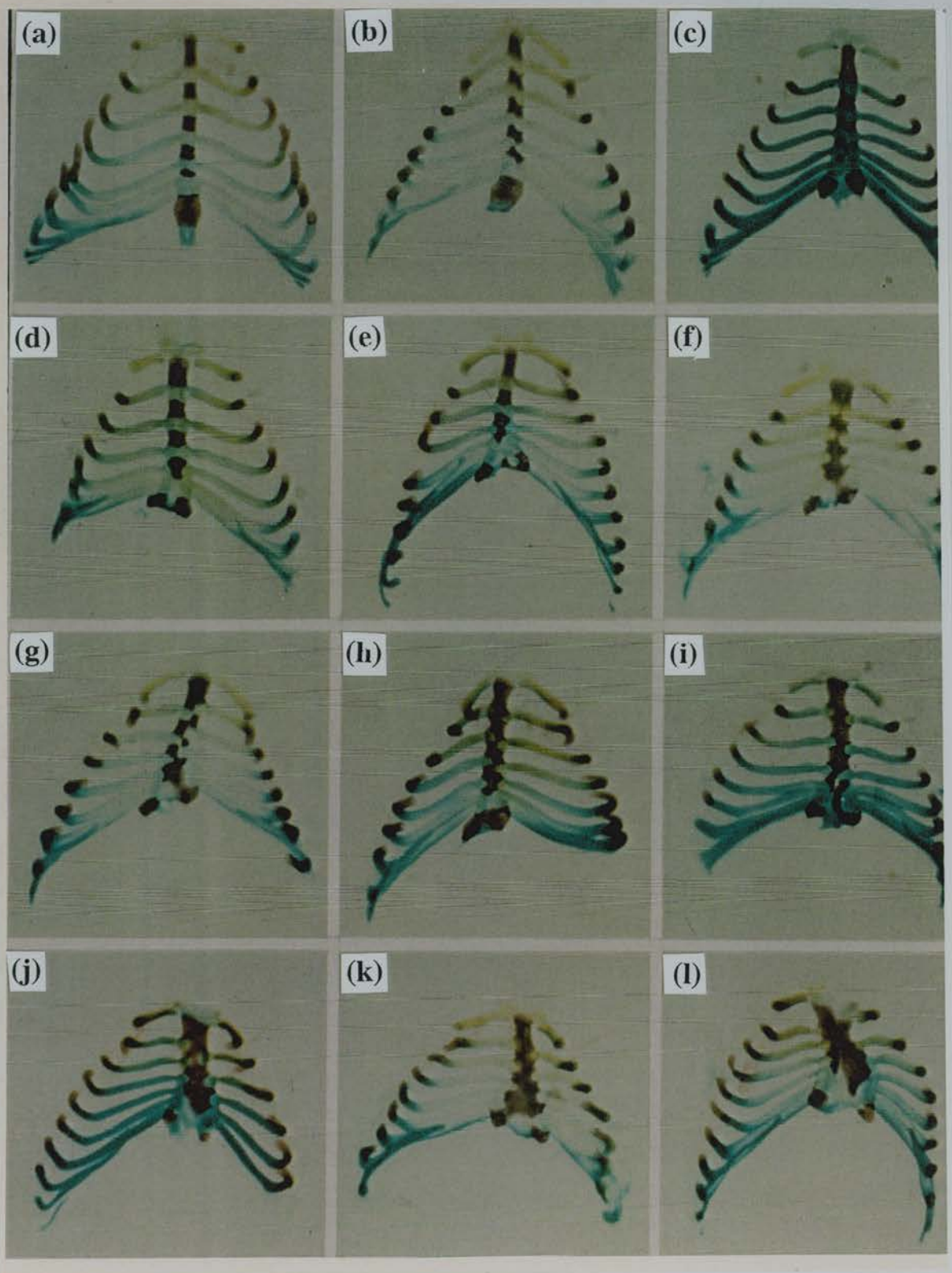
(g). The chest wall is markedly asymmetric, and each of the sternebrae appears to have 2 small centres which are obliquely related to each other and partially fused together. As in (f), the lower sternebral centre is fused to the left xiphisternal centre.

(h). The chest wall is asymmetric. Four sternebral centres are present but all are fused to each other to give a single chequered-shaped unit. The 2 xiphisternal centres have fused together across the midline.

(i). The manubrial and upper 2 sternebral centres have fused together. The 3rd sternebral centre is duplicated, and the right side has fused to the 2nd sternebral unit, while that on the left side has fused to the left xiphisternal centre via a bridge of ossification. A 2nd distinct centre is present on the right side of the xiphisternum.

(j). The manubrium, sternebral and xiphisternal centres have fused together to produce a single unit, the only separate entity being the left xiphisternal centre.

(k). The manubrial and sternebral centres have fused together to produce a grossly abnormal ossifying unit. The latter is associated with 2 separate xiphisternal centres.


(a)
(b)
(c)
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(e)
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(g)
(h)
(i)
(j)
(k)
(l)

Chapter 4: Transient bradycardia following amniotic sac puncture in the mouse

4.1 Introduction

A mouse model of amniotic sac puncture, which exhibited a high incidence of craniofacial and limb abnormalities, has been established (chapter 2). The abnormalities resemble those seen in the oromandibulofacial limb hypogenesis (OMFL) syndrome occasionally encountered following chorionic villus sampling (CVS) carried out during early human pregnancy; among them, limb abnormalities have been investigated in detail with reference to skeletal elements as well as deformities of the wrist and ankle joints (chapter 3).

Whilst two plausible mechanisms, namely "intrauterine constraint" and "vascular disturbance", had been advanced to explain the abnormalities encountered (Poswillo, 1966, 1968; Kennedy & Persaud, 1977; Miller, 1983; Houben, 1984; Kaufman, 1994; MacIntyre *et al.*, 1995; for details also see section 2.4.1.1), no further evidence in regard to the teratogenesis following amniotic sac puncture have been forthcoming. This was mainly due to the fact that only at day 19 of gestation the embryos previously subjected to amniotic sac puncture were analysed, as reported in chapters 2 and 3. By d 19, early signs of uterine constriction or vascular disturbance might have disappeared. Critical changes might have occurred immediately or shortly after amniotic puncture and not been recognized.

In the rat, craniofacial defects similar to those observed in the OMFL syndrome had previously been induced experimentally following vascular disruption of the pregnant uterus (Brent, 1990; Brent and Franklin, 1960). Comparable limb defects had also been observed following uterine trauma and clamping of the uterine arteries for 45 minutes (Webster *et al.*, 1987). Furthermore, extensive subcutaneous haemorrhages, a condition termed ecchymoses, with or without large blebs filled with clear fluid or blood, were noticed 15 minutes to 48 hours after amniotic sac puncture in the rat (Houben 1984; Kennedy and Persaud, 1977; Love and Vickers, 1972; Singh and Singh, 1973). Histological analysis of these lesions confirmed that vascular damage had been induced; haemorrhage, vascular engorgement and tissue disruption, and evidence of interstitial tissue oedema were all seen, supporting the involvement of vascular interruption.

In the human, two major mechanisms have been proposed to explain the limb defects seen following early CVS: (1) Entrapment of one or more distal extremities may occur in the exocoelomic gel located between the amnion and the chorion. This is said to be present before day 66, at the time corresponding with the obliteration of the exocoelomic space, and may result in temporary immobilisation of the trapped part(s) with subsequent ischaemia and necrosis (Burton *et al.*, 1992; Shepard *et al.*, 1991). (2) Vascular

disruption may occur in response to the removal of a small chorionic villus sample, causing either fetal hypotension, or hypoperfusion and anoxia due to fetal blood loss, or thrombus formation at the site of biopsy with distal embolisation (Brent, 1993; Burton *et al.,* 1992; Holmes, 1993; Kaufman, 1994; Report of the NICHHD workshop, 1993; Firth *et al.*, 1994).

The evidence so far available, based on clinical observations, strongly favours a vascular aetiology in causing the limb anomalies/deformities encountered in a small proportion of cases following CVS during early pregnancy. Long before the suggestion of any possible relationship between CVS and limb anomalies had been raised, four fetuses that had not previously been exposed to CVS were reported with limb reduction defects, believed to be due to fetal vascular occlusion (Hoyme *et al.,* 1982). Using embryoscopy, Quintero *et al.* (1992) subsequently provided direct evidence of a possible link between placental trauma and haemorrhagic lesions in human fetal limbs. However, to date, no direct evidence of a relationship between a transient period of fetal bradycardia and CVS has been reported. This might be due to the low incidence of orofacial and limb defects following this procedure, the small sample sizes in most studies (Brezinka *et al.,* 1995; Hibbard *et al.,* 1994; Zoppini *et al.,* 1993), or because the studies had been performed at periods mostly after day 66 of gestation (Kofinas *et al.,* 1995). Another possible problem might arise because of the *delayed* onset of bradycardia which might not be noted if monitoring of fetal heart rate were made only in the *immediate* post-CVS period (Burton *et al.,* 1992).

Burton *et al.* (1992) performed ultrasound examination on the fetus and placenta immediately after CVS in 294 cases in order to establish whether bradycardia was induced *immediately* following this procedure. While none of the fetuses in this study displayed evidence of bradycardia or evidence at that time of morphological abnormalities, four fetuses did show transverse limb reduction defects at the time of birth.

Hibbard *et al.* (1994) investigated, using Doppler velocimetry, fetal umbilical arterial blood flow and heart rate directly after CVS on 21 consecutive individuals who underwent this procedure during the first-trimester of pregnancy, but in all cases failed to discover any significant difference in values before and *immediately* after CVS when the data were analysed by gestational age, villus biopsy sample size, method (ie. transabdominal or transvaginal CVS), number of aspirations, placental location, or operator.

Brezinka *et al.* (1995) studied the Doppler flow velocity waveform recording in the fetal ductus venosus, umbilical artery and maternal serum alpha-fetoprotein (MSAFP) level in a cohort of 36 women of advanced maternal age who received transabdominal CVS (TACVS) between 11 to 13 gestational weeks. In one of the three cases in this series in which a high post-CVS MSAFP level was observed, bradycardia and other abnormal cardiac responses were reported following TACVS, and resulted in fetal loss 1 week later. No information was provided on the normality or otherwise of the limbs of this fetus. Kofinas *et al.*

(1995) examined 42 patients in which CVS was undertaken between 9.5 and 12 weeks of gestation, and found no evidence of bradycardia following CVS. In all of these cases, however, vascular analysis was performed well beyond the day 56 to 66 "window" when limb abnormalities are most likely to be induced.

Based on these findings and the nature of the limb defects seen, it was proposed that these lesions might have resulted from a transient period of fetal bradycardia, possibly due to compression of the uterus and the extra-embryonic membranes (Brezinka *et al.*, 1995; Burton *et al.*, 1992; Hibbard *et al.*, 1994; Kofinas *et al.*, 1995). Only one experimental report, however, indicated the presence of fetal bradycardia following amniotic sac puncture in the rat (Houben, 1984), though no detailed findings were presented.

This chapter intends to explore further the proposed vascular interruptions by looking at the embryonic heart rate, incidence of cardiac arrhythmias and heart "block", as well as the extent of ecchymoses following amniotic sac puncture. The difference between the vascular interruption in the mouse amniotic sac puncture model and the clinical findings following CVS in the human will also be discussed.

4.2 Materials and methods

4.2.1 Mouse (details see section 2.2.1)

4.2.2 Definition of experimental and control groups

Four groups of pregnant mice were investigated in this study: (a) a "non-experimental" control group without anaesthetics; (b) an "anaesthetics-injected" control (or "anaesthetics only" control) group without midline laparotomy (thus, without amniotic sac puncture); (c) an "internal" control group that was subjected to midline laparotomy without puncture; and (d) an "experimental" group that was subjected to both midline laparotomy and amniotic sac puncture.

Females in group (a) were autopsied in the morning on either day 12, 13, 14 or 15, in order to provide baseline information on embryonic heart rate at these various times during normal fetal development. Females in group (b) were anaesthetised according to the weight-related schedule described below, but were not exposed to any operative procedure. Females in group (c) and (d) were given a general anaesthetic, i.e. an intra-peritoneal injection of 0.016 ml of a 2.5% solution of Avertin per gram of body weight (Hogan *et al*, 1986). A midline laparotomy was then performed and one of the uterine horns exteriorized. Embryos exposed to amniotic sac puncture in the exteriorized uterine horn were classified in group (d), while embryos in the contralateral horn were undisturbed and acted as "internal" controls in group (c).

Amniotic sac puncture and was applied to the "experimental" horns as described in section 2.2.2.3.

Similarly, no attempt was made to select either the right or left uterine horn. The most easily accessible horn was exteriorized on each occasion, as we have previously observed in chapter 2 that the ratio of left:right horns using this approach is close to unity (Chang *et al.*, 1996).

4.2.3 Autopsy of females

Females in group (a) were autopsied between 0900 to 1100 on days 12 to 15 of pregnancy and embryos were isolated from both uterine horns. Females in group (b) were autopsied on the morning of day 13 of pregnancy at 30 minutes, 2 hours, and 4 hours following the anaesthetic injection. In groups (c) and (d), females were autopsied on the morning of day 13 of pregnancy. For group (c), studies were carried out only at 2 hours following the anaesthetic injection. For group (d), studies were carried out at 2 hours and 4 hours following the anaesthetic injection.

Uterine horns were opened carefully and rapidly, in order to avoid of causing damage to the placentas, the embryos, or their extraembryonic tissues. Each embryo with its attached placenta and associated extraembryonic tissues was kept as an intact unit and immersed in normal saline buffered with 25 % fetal calf serum contained in a 15 ml Falcon tissue culture tube. The whole preparation was maintained at 37°C in a water bath until heart rate determinations were carried out.

4.2.4 Counting heart rate (beats/min)

An attempt was initially made to use a miniature ECG device and colour detection video system (Dyson *et al.,* 1995) but the sensitivity of the system employed proved to be inadequate. Under the circumstances, either the intact unit indicated above (group (a)) or the isolated embryos (still attached to their extra-embryonic tissues) were explanted into tissue culture medium maintained at 37°C on a heated stage in a heated cabinet. Embryos were immediately transferred to the tissue culture tube (Falcon) containing medium, and retained in the water bath at 37°C until required. One embryo at a time was placed onto a tissue culture dish containing medium, and viewed by time-lapse cinemicrography for 75 to 90 seconds. The embryonic heart beat remained stable for about 15 minutes in the 37°C water bath. Two operators were always working together to ensure the embryonic heart beat was measured and its rate established. By this means, the heart rate (beats/minute) could be accurately determined (an average of three counts from the recordings was used for all subsequent studies).

In embryos isolated from females in group (a), the embryonic heart rate could be monitored indirectly by analysing the pulsation of the umbilical cord or the movement of the body (Suzue, 1994) through the intact extraembryonic membranes. Following amniotic sac puncture, it was necessary to expose the embryo in order to view the pulsation of the heart chambers more precisely. Care was taken not to damage the major blood vessels which connected the embryo and the placenta in order to maintain an intact embryonic circulation. Embryos in which the extraembryonic membranes were ruptured during their isolation from females were discarded, and data from embryos which displayed either arrhythmia or evidence of a heart "block" were also excluded from the study. Note was taken, however, of the incidence of embryos which belonged to one or other of these two categories.

4.2.5 Statistics

At least 20 embryos from three different females were investigated in each sub-group, and paired t-test was applied between/within groups.

4.3 Results

4.3.1 Embryo viability

In the normal control series (group a), an unexpectedly high incidence of death was seen in the day 12 embryos, while no dead embryos were observed when a similar analysis was performed on day 13, 14 or 15. It is likely that the high incidence of death represents a technical difficulty in association with the system of analysis, rather than a real finding.

In the "anaesthetic only" series (group b), the incidence of dead embryos increased slightly from a value of 17 % observed at 30 minutes after the anaesthetic injection, to 20 % and 25 % at 2 hours and 4 hours, respectively.

In the "internal" control series (group c), no dead embryos were observed when analysed at 2 hours after anaesthetic injection. This contrasts with the situation observed in the "experimental" series (group d) where the incidence of dead embryos observed at 2 hours and 4 hours was 22 % and 51 %, respectively.

4.3.2 Heart rate studies

4.3.2.1 Embryonic heart rate during normal development

The results of the embryonic heart rate from the "non-experimental" control series (group a) are presented in Table 4.1. Only recordings from those embryos with regular rhythm, i.e. in sinus rhythm, were counted and included in Table 4.1, though the incidence of arrhythmias was also noted (see section 4.3.4).

In Table 4.1, it appeared that the mean embryonic heart rate was at around 100 - 101 beats/minute in day 12 and 13 embryos, being slower as compared to that of day 14 and 15 embryos where the embryonic heart rate was at around 110 - 112 beats/minute. The difference, however, was not significant ($p > 0.5$). A wide range of heart rate within each sub-group was also noted.

4.3.2.2 Embryonic heart rate following anaesthesia without either laparotomy or amniotic sac puncture

The results of the embryonic heart rate at different time intervals (30 minutes, 2 hours, and 4 hours) following anaesthesia without laparotomy and amniotic sac puncture (group b) are presented in Table 4.2. At 30 minutes after anaesthesia, a reduction in the heart rate was seen, i.e. mean heart rate at 52.7 beats/ minute (± 2.79) in contrast to the normal mean heart rate at 101.7 beats/minute (± 4.06; see table 4.1) of day 13 normal embryos in group a. The reduction was reversed to control levels after 2 hours, when the mean heart rate was increased to 103.1 beats/minute (± 4.98). At 4 hours following anaesthesia, while a slight reduction in the heart rate was observed, the rate was not significantly different from the value obtained at 2 hours, and in the control series.

4.3.2.3 Embryonic heart rate following anaesthesia and amniotic sac puncture

The results from the "internal" control series (group c) and the experimental series (group d) are shown in Table 4.3. A significant reduction in the heart rate was observed in the experimental series (group d) when this was determined at 2 hours following anaesthesia, but normal levels were achieved in this group by 4 hours following anaesthesia. This contrasts with the findings in the "internal" control series (group c), where normal levels were achieved by 2 hours following the anaesthetic.

4.3.2.4 Maternal heart rate

While the ECG recordings were not sufficiently sensitive to provide information on embryonic heart rate, it was observed that the maternal heart rates (at embryonic gestation day 13 and during the period when the pregnant females were deeply anaesthetised) were within the range of 375 to 425 beats/minute.

4.3.3 Ecchymoses seen in control and experimental groups

No evidence of ecchymoses was seen in either the non-experimental control series (group a) or in the anaesthetics only series (group b). In group c (the 'internal' control series), *occasional* evidence of ecchymoses was seen in the head and trunk region at 2 hours and 4 hours following anaesthesia. The ecchymoses, however, had invariably disappeared when the embryos were examined early on the following day (i.e. day 14). In the experimental series (group d), in contrast to that in group c, extensive areas of ecchymoses in the head, trunk and limbs were *invariably* present. Evidence of cutaneous bruising was still apparent when the embryos were examined early the following day, though no evidence of ecchymoses was observed on the afternoon of day 14.

4.3.4 Incidence of cardiac arrhythmias and heart "block"

Careful note was taken to determine whether the heart beats were regular or otherwise. The cases of regular heart beating were included in Tables 4.1 - 4.3. Cases of irregular heart beating, i.e. arrhythmias, were not counted in Tables 4.1 - 4.3, but were recorded. Two principal types of arrhythmias were recognised, namely sinus arrhythmia where periods of rapid regular beats were interposed with periods of slower regular beats, and embryos where there was clear evidence of an atrio-ventricular (A-V) block.

In the cases of sinus arrhythmia, the heart rate was not determined, even though this probably represented a normal variant, and may not be harmful to survival and, in all probability, reverts to sinus rhythm. It then follows that the embryos that require further detailed analysis are those in which an A-V block was induced, as these are less likely to revert to sinus rhythm, and may well die.

When the heart rate was determined in the living embryos in the normal series (group a) on the morning of either day 12, 13, 14 or 15 of pregnancy, out of a total of 119 embryos studied, in 24 cases there was evidence of an arrhythmia; in all but one case this consisted of sinus arrhythmia, i.e. periods of rapid regular beats interposed by periods of slower regular beats. In a single case, evidence of an A-V block was apparent. The detailed findings in relation to this group are presented in Table 4.4.

In the anaesthetic only series (group b), the incidence of dead embryos in the non-damaged groups studied at 30 minutes, 2 hours and 4 hours was 17 %, 20 % and 25 %, respectively. While the heart rate increased from the mean value recorded at 30 minutes, and had achieved a normal value by 2 hours a moderate incidence of sinus arrhythmias and A-V blocks was observed; the incidence of dead embryos ranged from 17 to 25 % (see Table 4.4).

In the "internal" control series (group c), all embryos were alive when examined at 2 hours after the anaesthetic injection. This contrasts with the findings in the "experimental" series (group d) where the incidence of dead embryos was 22 % at 2 hours, and 51 % at 4 hours after the anaesthetic injection. The high incidence of embryonic death in the latter group was similar to the findings from the earlier analysis of the experimental fetuses studied on day 19 of pregnancy where the incidence of embryonic losses was 47 %. In the "internal" control series, the comparable figure was 3 % (MacIntyre *et al.*, 1995).

In the "internal" control series (group c), the incidence of sinus arrhythmias and A-V blocks observed at 2 hours was significantly lower than in the comparable anaesthetic only group - though no explanation for this observation is presently forthcoming.

In the anaesthetic only group, a low incidence of A-V block is recognised at 30 minutes, it increases by 2 hours and decreases slightly by 4 hours. At the same time, an increased incidence of dead embryos is seen in the 4 hour compared to the 2 hour group.

In the "experimental" series, the incidence of A-V block at 2 hours, though relatively high (17 %), diminishes to 4.2 % at 4 hours, but this is more than compensated by the increased incidence of dead embryos seen at 4 hours (51 %) compared to that seen at 2 hours (22 %).

4.4 Discussion

4.4.1 The cause of transient bradycardia in the experimental series

Results in the present study showed that the embryos in the "experimental" series (group d) had significantly slower mean heart rate in comparison to that in the 'internal' control embryos (group c). It also took a significantly longer period of time for the embryos in group d to revert their mean heart rate to normal levels, as compared to those "internal control" embryos (group c) in the contralateral uterine horn that were subjected to anaesthesia alone. It was also shown, in group b, that while exposure to anaesthesia alone was sufficient to induce a transient significant reduction in the heart rate, the heart rate was reverted

to normal levels only 2 hours following the anaesthetic injection. In the "experimental" series (group d), however, it took a total of 4 hours to revert the heart rate to normal levels.

The simplest, and probably the most obvious, explanation for this finding would appear to be that the loss of a substantial volume of amniotic fluid from this compartment had a constricting effect on the embryo. As a result of the constriction effect, the general blood flow with the embryo might have become substantially reduced and in some cases circulatory stasis occurred, inducing a transient but more prolonged period of bradycardia over and above that already induced by exposure to anaesthesia alone. Another possible cause of the transient bradycardia might have been from the vasoconstriction factors such as histamine and prostaglandins as discussed in chapter 2. These factors, having a potential role in causing a general circulatory stasis, were released only in the amniotic sacs that were subjected to puncture. They probably contributed to the prolonged period of bradycardia in addition to the effect from anaesthesia.

4.4.2 The effect of transient bradycardia on limb development

A transient period of bradycardia, be it from anaesthesia or puncture or both, is likely to cause hypoperfusion of tissues in the developing embryo. The longer the period of bradycardia is, the more evident the effects of hypoperfusion may be seen. This is evidently indicated by the extensive ecchymoses (or cutaneous bruising) that had been invariably seen at different sites on the surface of the embryos from both the "internal" control series and the "experimental" series. Furthermore, while minimal evidence of bruising was apparent in the "internal" control series at 2 and 4 hours after anaesthesia, and had invariably disappeared by the following morning, extensive areas of bruising were still evident in the "experimental" series, either at 2 or 4 hours after anaesthesia or in the following morning. The results of such a prolonged period of ecchymoses (hypoperfusion) will affect embryonic development. This explains the extremely high incidence of abnormalities in the "experimental series", in contrast to the minimal outcome of abnormalities within the "internal" control series.

The fact that the forelimbs and the hindlimbs have been found to exhibit many more abnormalities than the superficial parts of the head and trunk region (where virtually no gross abnormalities were seen; see chapter 2) also supports the hypothesis that ecchymoses (and thus hypoperfusion) may play a role in causing abnormalities. Although ecchymoses have been found in the superficial parts of the head and trunk region, they probably did not remain long enough to exert any long-term damage. Alternatively, the superficial parts of the head and the trunk in the day 13 developing embryos may not be as sensitive as the limbs in their response to ecchymoses. This is particularly implied by the fact that the apical ectodermal region (AER) resides in the superficial distal part of the developing limb.

Although a postural basis of teratogenesis for the limb abnormalities cannot be excluded (see chapter 2), it is not likely to account for the characteristic abnormalities seen involving the digits, where the principal damage appears to arise as a consequence of failure of separation of the digits rather than damage to the skeletal system of the digits (Chang *et al.,* 1996). In the light of the transient bradycardia observed here, the hypothesis may be advanced to suggest that hypoperfusion of the peripheral part of the developing limb bud, which probably interferes with the normal pattern of programmed cell death (i.e. apoptosis) that occurs in the interdigital zones. Alternatively, an abnormal degree of cellular proliferation or tissue regeneration may be induced in these sites, possibly because of overexpression of certain patterning genes, or due to the presence of growth factors which have been brought to the vicinity by macrophages during their clearing of the necrotic tissues (Hopkinson-Woolley *et al.,* 1994).

4.4.3 Relevance of transient bradycardia in the mouse model to clinical finding in the CVS

Having observed the transient bradycardia *beyond that observed as a consequence of exposure to the anaesthetic* in the animal model, one has to ask why it had not previously been reported in the various clinical studies that have been carried out to determine whether this also occurs following CVS ?

There are several explanations for this difference:

(1) This phenomenon is only likely to occur in those instances where inadvertent puncturing of the amniotic sac occurs during CVS, and is followed by an uncontrolled loss of amniotic fluid, *when this procedure is carried out during early pregnancy.* The relative proportion of amniotic fluid loss following ASP is likely to be far greater in the mouse model than in the human CVS.

(2) The overall incidence of CVS-induced abnormalities, though significant, is still extremely small, being in the order of 0.09 to 1.7% (Schloo *et al.,* 1992; Firth *et al.,* 1991a, respectively), with the highest incidence observed when this procedure is carried out between days 56 to 66 of pregnancy. In order to recognise these cases, it would be necessary to monitor very large numbers of *consecutive* cases where these criteria were met. In the few published series in which monitoring had been undertaken (Brezinka *et al.,* 1995; Hibbard *et al.,* 1994; Kofinas *et al.,* 1995; Zoppini *et al.,* 1993), the overall sizes of the groups studied have all been relatively small, but probably more importantly, the age groups studied have only contained a few very early and therefore particularly susceptible pregnancies. When later stages of pregnancy are exposed to CVS, the risk of inducing craniofacial and/or limb defects even following the inadvertent puncturing of the amniotic sac is substantially reduced.

(3) The experimental animal model used in this study is designed to maximize the incidence of craniofacial and/or limb abnormalities induced by amniotic sac puncture. Uncontrolled amniotic fluid leakage is regarded as a prerequisite for successful ASP. Therefore, any effect on embryonic heart rate induced by the experimental procedure might be expected to be observed.

(4) In our earlier studies reported in chapters 2 and 3 (Chang *et al.*, 1996; MacIntyre *et al.*, 1995), it has been observed that 27 % of the mouse embryos that had been subjected to amniotic sac puncture on day 14 of pregnancy, and survived to day 19, developed a cleft palate, and 39 % had one or more morphologically abnormal limbs. This stage of mouse gestation approximately corresponds to Carnegie Stages 20 to 23 or days 50/51 to 56/57 days post-ovulation of human pregnancy (O'Rahilly and Müller, 1987), and is similar to the timing during pregnancy when the *earliest* cases of CVS were carried out in the various clinical trials indicated previously. The incidence of palate and limb abnormalities was significantly increased, however, in our model system when amniotic sac puncture was carried out on day 13 of pregnancy, rising to 35 % and 61 %, respectively. The overall incidence of abnormalities of one or other system in the day 13 group was over 70 %. It is for this reason that we have concentrated our attention on the day 13 group.

When amniotic sac puncture is carried out in the morning on day 13 in the mouse, this approximately corresponds to Carnegie Stage 17/18 of human embryonic development, equivalent to 41 to 44 post-ovulatory days, or during the 6th week of pregnancy. While this is somewhat earlier than the timing when CVS is normally carried out in the various clinical trials cited previously, the combination of an earlier timing and the fact that there is deliberate puncturing of the amniotic sac probably explains why such a high incidence of abnormalities, particularly of the limbs, is seen in this model system. Conversely, the slightly later timing, and only the occasional and inadvertent puncturing of the amniotic sac would appear to explain the relatively low incidence of abnormalities encountered following CVS in clinical practice. The fact that the range of limb, but particularly digital, abnormalities encountered in the model system resembles some of those seen following CVS suggests that the underlying mechanism in the two situations may be similar.

We only very rarely observe transverse limb reduction defects in this model. Their aetiology, mostly unascertained, though occasionally resulting from the effect of amniotic bands (Keeling and Boyd, 1993), is likely to be different from the characteristic type of limb abnormalities reported here and previously (Chang *et al.*, 1996; MacIntyre *et al.*, 1995).

Table 4.1 Embryonic heart rate during normal development. Heart rate (beats per minute) of day 12~15 mouse embryos*^

Day of pregnancy	Mean (S.E.M.)	Range	Sample size (n)
12	100.2 (2.85)	69 - 133	22
13	101.7 (4.06)	79 - 132	21
14	110.1 (3.93)	72 - 144	26
15	112.1 (5.14)	70 - 155	22

*Heart rate determined only on embryos with regular heart rate (ie in sinus rhythm)
^The significance is set at $P<0.05$; for statistical differences between/within groups, see text
†For definition of these groups, see text

Table 4.2 The effect of general anaesthesia on the heart rate of day 13 mouse embryos (i.e. "anaesthetic only" group)*^ The embryonic heart rate (beats per minute) was detected at intervals following an intraperitoneal injection of Avertin (a general anaesthesia, details see section 2.2.2.1) to their mothers.

Time after anaesthetic	Mean (S.E.M.)	Range	Sample size (n)
30 min	52.7 (2.79)	25 - 85	33
2 hrs	103.1 (4.98)	52 - 147	21
4 hrs	92.5 (6.20)	25 - 129	23

*Heart rate determined only on embryos with regular heart rate (ie in sinus rhythm)
^The significance is set at $P<0.05$; for statistical differences between/within groups, see text
†For definition of these groups, see text

Table 4.3 The effect of amniotic sac puncture on the heart rate of day 13 mouse embryos*^ The embryonic heart rate (beats per minute) was detected at intervals following the ASP.procedure.

Time after anaesthetic	Mean (S.E.M.)	Range	Sample size (n)
"internal" control 2 hrs	97.6 (5.52)	53 - 143	21
"experimental" 2 hrs	61.4 (5.05)	22 - 103	23
"experimental" 4 hrs	106.2 (7.62)	62 - 152	21

*Heart rate determined only on embryos with regular heart rate (ie in sinus rhythm)
^The significance is set at $P<0.05$; for statistical differences between/within groups, see text
†For definition of these groups, see text

Table 4.4 : Summary of the distribution of mouse embryonic heart rates*^ (beats per minute) during normal development, at intervals following a general anaesthetic (Avertin), and after amniotic sac puncture

Group		Litters	Sample size (N+R)§	Damaged during dissection	Embryos analysed							
					Total dead embryos	(%)	Total living embryos	(%)	Heart rate analysed: Normal rhythm	(%)	Arrhythmias†	(%)
Normal	Day 12	5	49+1R	12	10	(27%)	27	(73%)	26	(96%)	1	(3.7%)
	Day 13	3	33	6	0		27	(100%)	21	(78%)	5	(19%)
	Day 14	5	52	15	0		37	(100%)	26	(70%)	11	(30%)
	Day 15	5	40+3R	12	0		28	(100%)	22	(79%)	6	(21%)
Anaesthetic only	30 min	6	58+2R	11	8	(17%)	39	(83%)	33	(85%)	5	(13%)
	2 hrs	5	46+3R	5	8	(20%)	33	(81%)	21	(64%)	6	(18%)
	4hrs	4	38+5R	2	9	(25%)	27	(75%)	23	(85%)	1	(3.7%)
"internal" control	2 hrs	4	23*	0	0		23	(100%)	21	(91%)	1	(4.4%)
"experimental"	2 hrs	8	40**	3	8	(22%)	29	(78%)	23	(79%)	1	(3.5%)
"	4 hrs	11	53**	4	25	(51%)	24	(49%)	21	(88%)	2	(8.3%)

Group		Embryos analysed: Heart rate analysed: A-V block‡	(%)
Normal	Day 12	0	
	Day 13	1	(3.7%)
	Day 14	0	
	Day 15	0	
Anaesthetic only	30 min	1	(2.6%)
	2 hrs	6	(18%)
	4hrs	3	(11%)
"internal" control	2 hrs	1	(4.4%)
"experimental"	2 hrs	5	(17%)
"	4 hrs	1	(4.2%)

*Heart rate determined only on embryos with regular heart rate (ie in sinus rhythm)
^The significance is set at $P<0.05$; for statistical differences between/within groups, see text
§N = total embryos in both uterine horns R = total number of resorption sites in both uterine horns
†In the majority of cases, the heart rate consisted of periods of rapid regular beats, interposed by periods of slower regular beats, termed sinus arrhythmia.
‡Atrio-ventricular block
*total embryos in the "internal" control horns
**total embryos subjected to amniotic sac puncture

Chapter 5: Analysis of interdigital spaces during mouse limb development at intervals following amniotic sac puncture

5.1 Introduction

5.1.1 Brief review of previous findings

Investigations in chapter 2 revealed that the teratogenic effect of amniotic sac puncture on mouse embryos induced abnormalities in the palate, limb and tail. In chapter 3, limb and other postcranial skeletal defects induced by amniotic sac puncture in the mouse were further investigated, with detailed analysis on the chondrification and ossification of the limb skeletal elements, the wrist and ankle joints, and the comparison of soft tissue anomalies with those of the underlying skeleton. Using whole-mount double-staining techniques (detailed in chapter 3), it has been revealed that the syndactyly induced by amniotic sac puncture exclusively involves the soft tissues. This is in general agreement with other skeletal studies of affected limbs in children and in advanced fetuses of experimental animals that have been analysed by radiography (Kino, 1975) and histology (Love and Vickers, 1972; Houben, 1984). In the mouse embryos subjected to amniotic sac puncture, even in cases of adactyly, it was observed that the soft tissues were affected (leading to syndactyly) whilst all the skeletal elements were present (see chapter 3; Chang *et al.*, 1996). Syndactyly, therefore, in these cases, appears exclusively to be due to the failure of separation of the soft tissues in the interdigital zones, which is a normal patterning process associated with digit formation.

As indicated in previous chapters, various hypotheses have been proposed to account for the pathogenesis of the various abnormalities observed in mouse embryos subjected to amniotic sac puncture. For example, in chapter 2, it has been suggested that intrauterine compression of the embryo by the extraembryonic membranes and the uterine muscles following the loss of amniotic fluid might cause immobilisation and postural remodelling of the embryo, leading to the range of abnormalities (e.g. of the palate and limbs) seen. This not only explains the craniofacial defect (details see section 1.3.3.1), but also served as the most plausible explanation for the limb abnormalites (details see section 1.3.3.4). Equally, following CVS, fetal blood loss, or thrombus formation within vessels in the vicinity of the biopsy site, could result in either fetal hypotension, or embolisation, hypoperfusion and anoxia of the extremities (Kaufman, 1994).

In chapter 4, further investigations have revealed that a transient period of fetal bradycardia may be demonstrated after amniotic sac puncture beyond that induced by anaesthesia alone. Mouse embryos were divided in three groups: embryos exposed to an anaesthetic only, embryos exposed to both an anaesthetic and amniotic sac puncture, and embryos exposed to neither of these procedures (ie the "non-experimental" control group). It was confirmed that a transient period of bradycardia occurred in only the first two of these three groups, with an *additional* delayed recovery in heart rate of about 1.5 - 2 hours in the second compared to the first group. The *additional* delay in heart rate recovery suggested an indirect vascular aetiology of the limb

abnormalities observed in embryos following experimentally-induced amniotic sac puncture. Transient bradycardia has also been noted both in human studies following CVS (N.Ginsberg, personal communication, cited by Firth *et al.,* 1991b) and in rat ASP studies (Houben, 1984), providing additional evidence that an *indirect* vascular aetiology may be involved in causing the limb abnormalities observed following CVS.

As introduced in section 1.3.3.2, craniofacial defects similar to those observed in the OMLHS syndrome can be induced in rat fetuses by vascular disruption (Brent and Franklin, 1960; Brent, 1990), and limb defects were also induced following uterine trauma and clamping of the uterine arteries for 45 minutes (Webster *et al.*, 1987). Similar findings were also observed when amniotic sac puncture was induced experimentally, with or without active withdrawal of amniotic fluid, at comparable gestational ages to those when CVS would normally be performed clinically (mouse: Trasler *et al.*, 1956; Walker, 1959; MacIntyre *et al.*, 1995; rat: Poswillo and Roy, 1965; Poswillo, 1966, 1968; Kendrick and Feild, 1967; DeMyer and Baird, 1969; Love and Vickers, 1972; Singh and Singh, 1973; Singh *et al.*, 1974; Kino, 1975; Kennedy and Persaud, 1977; Houben, 1980, 1984; Houben and Huygens, 1987). The findings from these studies indicated that vascular disruption was the most plausible explanation for the lesions observed. Cutaneous bruising ranging from mild petechiae to severe ecchymoses were noted on the head and trunk region, and/or on the limbs of experimental embryos, and appeared between 15 minutes and 48 hours following amniotic sac puncture. Histological analyses of ecchymotic regions of rat embryos have been carried out immediately after and at intervals following amniotic sac puncture, and have noted that within 2 - 3 minutes of carrying out this procedure, endothelial tears were already observed within the marginal vein in the interdigital regions as well as between the apical ectodermal ridge and its underlying mesenchymal tissues. Within thirty minutes, non-specific effects, such as venous congestion, vascular blebs, and periendothelial oedema, were first observed, though precartilaginous mesodermal condensations within the autopod did not appear to have been affected. At later times after experimental intevention, haemorrhages were observed in some of the interdigital areas of the affected limbs (Love and Vickers, 1972; Singh and Singh, 1973; Kennedy and Persaud, 1977; Houben, 1984; Houben and Huygens, 1987)). Thus the possibility exists that vascular disruption, secondary to a transient period of bradycardia, might play a role in inducing the limb abnormalities observed following amniotic sac puncture.

5.1.2 Hypothesis regarding soft tissue abnormalities in the interdigital zone

Since most of the digital anomalies observed following amniotic sac puncture in our model consisted of either syndactyly or adactyly, involving exclusively the tissues within the interdigital zones, various hypotheses have been proposed to explain the underlying mechanism(s) involved in the induction of these abnormalities (Chang *et al.*, 1996; Chang and Kaufman, 1997). For example, the abnormalities observed might have resulted from reduced/absent physiological cell death, increased cell proliferation (mitosis), or have resulted from the influence of growth factors which had been brought to the interdigital areas by macrophages when removing necrotic cells. Alternatively, syndactyly/adactyly might result as a consequence of maintenance of the undifferentiated status of mesenchymal tissues in areas in which apoptosis failed to occur.

5.1.3 Mouse amniotic sac puncture model as a reliable system for studying modifications in the interdigital zones

In the mouse model presented in this study (in chapter 2), the incidence of adactyly, syndactyly, or brachysyndactyly was about 74% in the embryos that survived following amniotic sac puncture (MacIntyre *et al.*, 1995). Thus, the mouse model provides a reliable system for studying cell death and mitotic activity in the interdigital zones in the experimental material. It also provides an opportunity for exploring the underlying mechanisms involved in the induction of digital abnormalities observed following amniotic sac puncture.

5.1.4 Aim of this chapter

To our knowledge, no *mouse* limbs previously subjected to amniotic sac puncture had been evaluated histologically. The aim of this chapter was therefore to compare findings in the mouse with those reported previously in the rat, particularly in the fate of the cellular components within the interdigital zone. In undertaking such a comparison, although others (see above) included information from the analysis of the limbs of dead embryos, such embryos were excluded from the present study.

5.2 Materials and Methods

5.2.1 Animals (details see section 2.2.1)

5.2.2 Production of "internal" control, "non-experimental" control and "experimental" groups

Three groups of pregnant mice were used in the present study: (a) a "non-experimental" control group; (b) an "internal" control group ; and (c) the experimental group. In group (a), pregnant mice which had not been subjected to either an anaesthetic or amniotic sac puncture were autopsied and their embryos collected at specific times during gestation, in order to provide baseline information relevant to these times. For groups (b) and (c), females were given a general anaesthetic (an intra-peritoneal injection of 0.016 ml of a 2.5 % solution of Avertin in normal saline per gram of body weight (Hogan *et al.*, 1986) at about 10 a.m. on day 13 of gestation. Once anaesthesia had been induced, a midline laparotomy incision was performed and one of the uterine horns exteriorized. The embryos of the exteriorized uterine horn were then exposed to amniotic sac puncture (the experimental group (c)). The embryos in the contralateral horn were left undisturbed, acting as "internal" controls (group (b)). The uterine horn subjected to amniotic sac puncture was chosen randomly, i.e. the most easily accessible horn being exteriorized on each occasion, as it has been observed in chapter 3 that the ratio of left:right horns using this approach is close to unity (Chang *et al.*, 1996). The ovary belonging to the "experimental" side, was identified, and the number and location of embryos and resorption sites recorded.

5.2.3 Amniotic sac puncture (details see section 2.2.2)

5.2.4 Time intervals following amniotic sac puncture

At 0.5, 4, 8, 12, 24, or 36 hours after amniotic sac puncture (ASP + h), the pregnant females were sacrificed by cervical dislocation. The uterine horns were removed and the embryos were dissected from within their extraembryonic membranes and separated into "internal" control and "experimental" groups according to the operating records. Only embryos that were alive at the time of isolation were studied further. For the "external" control group, time zero was taken as 10.00 a.m. on day 13 of gestation. In the experimental group, the exact timing in hours after ASP is provided in the form of (ASP + h), while in the "internal" control group, a similar system applies (O + h). In the "non-experimental" group, (NX + h) indicates the equivalent time when the "non-experimental" control limbs are isolated.

5.2.5 Histological processing

In each time group, the experimental and control embryos from more than one litter were fixed and their left fore- and hindlimbs were isolated and embedded in paraffin wax and serially sectioned. Histological processing involved the fixation of specimens in Bouin's solution for 10 to 24 hours according to the age of the embryos, following standard protocols (Kaufman, 1992). After fixation, the limbs were stored in 70 % alcohol until required. Fore- and hindlimbs from the left side, which we have previously reported generally display a slightly higher incidence of abnormalities on day 19 of gestation than limbs from the right side (MacIntyre *et al.*, 1995), were removed for appropriate processing. For all groups, a total of either 4 or 5 experimental and 3 control limbs were used for histological analysis in each time group. Serial coronal sections (when the limbs were oriented in the so-called anatomical position) were cut at a nominal thickness of 6 μm and stained with Erlich's haemotoxylin and eosin (H and E). The sections were examined and representative sections photographed using a Leitz Laborlux K photomicroscope.

5.2.6 Determination of sample size

Since the general incidence of *gross* limb abnormalities reported previously (MacIntyre *et al.*, 1995) was 22 % (right forelimb), 20 % (left forelimb), 35 % (right hindlimb), and 46 % (left hindlimb) when amniotic sac puncture was carried out during the morning on d 13 p.c., one could expect that the frequency of abnormalities observed at the cellular level would be substantially higher. It is for this reason that the sample sizes for each group indicated above were selected in the expectation that one or more samples from each group which display cellular abnormalities.

5.2.7 Method of cell number estimation in the interdigital zone

Analysis of interdigital zones to determine statistical differences between the various control and experimental groups was undertaken as follows. Dividing cells were readily recognized by the appearance of their mitotic figures (see Figure 5.1a). A total of 3 representative histological sections of either interdigital spaces II or III were photographed from each limb. In order to avoid analysing the same cells on more than one occasion, the first, fourth and seventh sections from a limb that had been serially sectioned at a nominal thickness of 6 μm were photographed using the x 25 objective of a Leitz photomicroscope and printed to a final magnification of x 530. Only the interdigital mesenchyme was analysed; the digital areas, surface epithelium and the immediately subjacent sub-epithelial mesenchymal tissues were excluded from this analysis. The area of interdigital tissue to be analysed was then measured using a Kontron image analysis system. Three regions measuring the equivalent of 50 μm x 50 μm, drawn on a transparent overhead, were randomly placed on the photomicrographs and the total number of intact nuclei within each square was counted, as formerly described by Tomasch and Malpass (1958). A knowledge of the number of nuclei scored in the sample squares and the total areas analysed, provides an accurate estimate of the total number of nuclei present in the sample areas analysed.

5.2.8 Determination of the degree of accuracy in cell number estimation

To estimate the degree of accuracy of this approach, the total number of nuclei in interdigital spaces from four randomly selected limb samples were manually counted. In these analyses, the estimated incidence was within the range 98 - 106 % of the value obtained by manually counting all of the nuclei present in the total areas sampled. In addition, a Pearson's χ^2 test was applied between the observed and the estimated values. No significant difference was found between these two sets of data, accordingly the estimation method described above was used. In this study, between 1500 - 2500 nuclei were scanned for each limb sample studied. The number of mitotic figures within the total interdigital area on each of the photomicrographs was also determined.

5.2.9 Observation of cellular morphology

Cellular morphology in the interdigital zones was examined in order to establish (1) whether there was evidence of vascular disruption, (2) to determine the incidence of cells in mitosis (i.e. mitotic index), (3) to determine the incidence of apoptotic cells, and (4) to establish the proportion of dead/necrotic cells.

Apoptotic cells were easily recognised at the light microscopic level based on the descriptions and illustrations provided in the literature (Saunders *et al.*, 1962; Glücksmann, 1965; Zakeri *et al.*, 1993). To distinguish between pyknotic cells (Figure 5.1b) and apoptotic cells (Figure 5.1c), histological evidence of the features illustrated by Hopkinson-Woolley *et al.* (1994) were sought and the account of necrosis/pyknosis described and

illustrated by Burkitt *et al.* (1993) was applied. Since a specific staining technique was not employed to investigate the incidence of apoptosis, the occurrence of these cells could not be unequivocally determined. However, as such cells were readily recognised, because of their characteristic histological morphology, a detailed *morphometric* analysis was not undertaken with regard to the incidence of apoptotic cells observed in the digital interspaces. Instead, a *descriptive account* of the incidence of pyknosis/apoptosis is provided in the various groups studied. Equally, although the pyknotic cells were also readily recognized based on their characteristic histological morphology (see Figure 5.1b), only a descriptive account is provided of their incidence in this study.

5.2.10 Statistical analysis of mitotic index in the interdigital zone between the control and the experimental groups

To test whether the mitotic indexes in the interdigital zone in the control and experimental groups were significantly different, the log likelihood ratio test and the χ^2 (chi square) test were undertaken as indicated in the appendix attached to the back of this chapter (W. Adams, personal communication).

5.3 Results

While appropriate numbers of fore- and hindlimbs from the left side were isolated at each of the time intervals indicated, technical difficulties were encountered which precluded the analysis of the ASP + 8h and 24h hindlimb samples. Distortion, principally in the form of flexion of the distal region of the footplate in many of the affected limbs meant that it was often not technically possible to obtain sections of the required orientation to display the detailed histological morphology of the digital interspaces. Because hindlimbs at day 13 of gestation develop about 12 hours more slowly than forelimbs (Kaufman, 1992), forelimbs only will be described unless any substantial difference between the fore- and hindlimbs needs to be addressed.

Although there is a brief transient episode of bradycardia observed in the "internal" control group (Chang and Kaufman, 1997), the forelimbs from this group were found to be histologically indistinguishable from those of the control group. Accordingly, only the "internal" control group is described below.

5.3.1 Histological observations

5.3.1.1 Control series

In the 0.5 h control group, the handplate exhibits a pentagonal shape. The pre-cartilaginous cells are aligned within the digital rays and the undifferentiated mesenchymal cells are located within the interdigital zones in association with the primitive capillary network. Immediately subjacent to the surface ectoderm at the distal margin of the handplate, there are 2 - 3 layers of compactly-arranged mesenchymal cells, termed the sub-

epithelial mesenchymal tissue/cells in the following text. The marginal vein is located beneath the sub-epithelial mesenchymal tissue. By contrast, in the footplate, there are usually 5 - 6 layers of sub-epithelial mesenchymal cells in this location. No indication of gross indentations in the interdigital zones are observed before about 4 hours (Figure 5.2a). In the 8-12 h groups, the pre-cartilaginous cells become increasingly aligned; by 24 h, the interphalangeal joints also show progressive evidence of differentiation, and by 36 h the proximal, middle, and distal phalanges of digits 2 - 5, and the proximal and distal phalanges of digit 1, are recognised. In the interdigital zones, the undifferentiated mesenchymal cells display no clear evidence of differentiation until physiological cell death (i.e. apoptosis) supervenes. The exact timing of these events varies between the different interdigital zones, being after about 4h within the peripheral area, or after 8 ~36 hours within the rest of the interdigital spaces. The depth of the indentation of the interdigital web increases as progressive cell death occurs (see Figure 5.2b).

5.3.1.2 Experimental series

Representative coronal histological sections through the forelimbs of experimental embryos previously subjected to ASP at 30 min (Figure 5.2c), 4h (Figure 5.2d) and 24h (Figures 5.2e and 5.2f) clearly demonstrate that a progressive *reduction in the degree of indentation normally seen in the interdigital zones* of matched control limbs occurs. Equally, by 24h after ASP, the marginal vein is (abnormally) still present in the experimental limbs. At this time it is also often possible to recognize gross abnormalities in the shape of the limb (see Figure 5.2f) and the presence of extensive areas of haemorrhagic disruption (Figure 5.2g).

In the most severely affected of the experimental limbs an extensive necrotic core often associated with haemorrhage and gross tissue disruption was characteristically seen which often obliterated the fine structure of the digital rays and interdigital zones (Figure 5.2g); in some instances, minimal evidence of interdigital indentations are observed. In the majority of the less affected limbs, the whole autopod appeared to become compressed, and this usually resulted in the presence of distorted cartilaginous and, later, skeletal elements, though the gross structure of the digital rays was similar to that seen in the matched control limbs.

The proximal-distal dimensions of the autopod of the experimental limbs were invariably observed to be smaller than those of developmentally matched controls; though this information was not quantified in the present study, it is planned that this might be undertaken if time permits.

5.3.2 The vascular events

5.3.2.1 Control series

Within the control limbs, the marginal vein initially runs along the peripheral margin of the handplate, being located between the sub-epithelial mesenchyme and the rest of the mesenchymal tissue of the autopod (Figures

5.3a and 5.3b). As the digits differentiate, the continuity of the marginal vein gradually becomes disrupted at the margins of the interdigital zones, being replaced by about 12h by a diffuse capillary network which spreads out within the interdigital zones. Although the diameter of the marginal vein remains fairly constant, indirect evidence of an increased blood supply was noted; the number of blood cells in the marginal vein in the forelimb in the 4 - 8 h groups increased by a factor of 2 - 3 as observed in the 12 h group. Nucleated primitive red blood cells are observed within the capillaries, there being no evidence of vascular congestion in either the marginal vein or in the capillary network during this period.

5.3.2.2 Experimental series

By contrast, in the experimental group, even as early as 0.5h after ASP the marginal vein was invariably seen to be dilated and appeared to be congested (Figures 5.3c and 5.3d), although it was unclear whether there was evidence of extravasated blood in the interdigital spaces, extending from the marginal vessels towards the base of the digits. The fact that it was often difficult to see the endothelial lining of the primitive capillaries precluded a definitive judgement on this point in most instances. The formation of enormous haematomas in the interdigital spaces appeared to be a late event, as none was found before ASP + 36 h, though extensive fluid filled spaces possibly containing serum were sometimes seen in these areas between 4 - 12h following ASP.

5.3.3 Apoptosis

Apoptotic bodies are characterised by their dense, pyknotic nuclei (see Figure 5.1.c). These are eventually engulfed by monocyte-derived macrophages in the majority of cases where apoptotic debris is characteristically found (Hopkinson-Woolley *et al.*, 1994). While it is fully appreciated that specific stains are now available to unequivocally demonstrate apoptotic cells, such cells are readily recognized in standard histological sections, because of their characteristic morphological features. Because of possible doubts as to the exact incidence of apoptotic cells in the interdigital spaces in our conventionally stained material, this component of our study could not be quantified, and is therefore based on the *qualitative* assessment of our findings.

5.3.3.1 Control series

During day 13 p.c. in the 4h, 8h and 12h control series, when the marginal vein is still intact, clusters of apoptotic bodies are found distal to and closely associated with the vein. During day 14 p.c., in the 24h and 36h groups, as massive cell death occurs in the interdigital space, large numbers of apoptotic bodies are observed in the interdigital zones, initially being seen in the distal part of spaces I and IV, then in spaces II and III. Subsequently, with the progressive loss of interdigital tissue, these bodies, while still found close to the interdigital margin, become more extensively distributed throughout the basal region of the space.

5.3.3.2 Experimental series

In the experimental autopods apoptotic cells may either be completely absent or dramatically reduced in number compared to those observed in the corresponding areas of control limbs. This reduction in the extent of apoptosis seen is first evident at 4 hours after ASP and becomes increasingly obvious over the next 24 - 36h period. At 24 hours after ASP, while the control limb possesses extensive evidence of apoptosis over a wide area in the interdigital space, only a limited number of apoptotic cells can be identified in comparable regions in the experimental limbs. In three cases (x2, ASP + 12h; x1, ASP + 36h), while apoptosis was absent in the distal part of the interdigital spaces, relatively few apototic bodies were observed in the proximal part of the interdigital space.

5.3.4 Mitosis

As far as cells in division is concerned, since there was no difficulty encountered in the recognition of mitotic figures, this component of the analysis could readily be quantified.

5.3.4.1 Controls series

In the control limbs, extensive evidence of cell division was seen throughout all regions of the autopod, within the surface ectoderm, digital rays and in the interdigital spaces, with the majority of the mitotic "bodies" being observed at the digital boundary and in the marginal (distal) part of the interdigital space. The mesenchymal cells within the interdigital spaces continued to divide until increasing evidence of apoptosis was observed, being initially seen in the distal region and subsequently in more proximal locations within the space. The detailed findings from the quantitative analysis of mitotic activity in the various control and experimental time groups studied are shown in Tables 5.1 and 5.2. The mitotic index was high at the beginning of the study (13.16 ± 0.84 per 1000 cells at ASP + 4h), and gradually declined to a very low level by the end of the study (2.36 ± 0.10 per 1000 cells at ASP + 36h).

5.3.4.2 Experimental series

Analysis of the mitotic index figures (see Table 5.2) revealed that at 4 hours after ASP the mitotic index in the experimental series was significantly lower than in the matched control group. The mitotic activity was then observed to return to control levels in the ASP + 8h group, and finally was observed to be significantly *higher* in the experimental limbs in the ASP + 12h, 24h, and 36h groups. In only the ASP + 36h group, of the 24h and 36h groups, where the experimental limbs could be divided into those that *appeared* to have either a grossly normal or abnormal morphology, the mitotic index of the mesenchymal cells in the interdigital zones of the grossly abnormal limbs was noted to be significantly higher than in the normal-looking limbs. In the ASP + 36h group, the mitotic index in the grossly abnormal limbs (6.19 ± 0.26) was observed to be

significantly higher than that in the normal limbs (1.49 ± 0.02). A relationship between vascular congestion and mitotic activity was also noted. In the ASP + 4h group, mitotic activity decreased in the presence of vascular congestion, and this was especially evident in the interdigital spaces. In the ASP + 8h and subsequent groups, vascular congestion and/or dilatation did not appear to interfere with mitotic activity; even in the most severe cases of vascular congestion, mitotic activity could be observed in the proximity of the marginal vein and its associated vascular network.

5.3.5 Dead cell and recruitment of macrophages

Dead cells may readily be distingushed from apoptotic bodies because of the presence in the former of dark, evenly-stained and spherical features (see Figure 5.1b) (Hopkinson-Woolley *et al.*, 1994). These can also be found as early as 0.5h after ASP, though similar cells were not usually observed in the control limbs at this time. No evidence of the recruitment of macrophages to the vicinity of these dead cells was observed, as these cells could not be unequivocally identified in these paraffin sections, and accordingly without the use of appropriate histochemically labelled cells (Hopkinson-Woolley *et al.*, 1994), we are unable at the present time to confirm this phenomenon.

5.3.6 Additional information gained from the analysis of hindlimbs

Analysis of the contents of the marginal vein in the non-experimental "external" control and "internal" control groups at 0.5h consistently revealed that exposure to anaesthesia alone, even at this time, induced a marked degree of congestion in this vessel (see Figures 5.4a and 5.4b). In all instances, in the ASP + 0.5h group, evidence of haemorrhage at various sites along the marginal vein was also seen (see Figure 5.4c), a phenomenon which was not observed in any of the forelimbs analysed at this time, though it was observed at later times. In one of the ASP + 36h group an example of a pre-cartilaginous "bridge" was observed in the distal part of the intervening interdigital zone which linked the distal phalanges of two of the central digits. This would, almost certainly, have given rise to a skeletal abnormality of the digits (a form of acrosyndactyly) in which two of the distal phalanges would be joined by a skeletal "bridge" (see Figures 5.2h and 5.2i).

5.4 Discussion

5.4.1 Inferefence with apoptosis is likely to be caused by vascular stasis that impedes apoptosis-triggering factor(s)

In this study, the apoptotic bodies observed in the control limbs are initially found only in association with the marginal vein. Thus, it is likely that apoptosis in the peripheral region of the interdigital space may be triggered by factor(s) such as oxygen released from the vascular system. Results in this study showed that the number of apoptotic bodies in the proximity of the marginal vein decreased shortly after amniotic sac puncture.

A few hours later, the apoptic bodies were almost completely absent in the interdigital zone. It was therefore hypothesized that venous congestion occurred due to the prolonged period of bradycardia following amniotic sac puncture (Chang and Kaufman, 1997), and that this inevitably led to a dramatic decrease in the oxygen supply to the target tissues as the arterial supply became impeded. Accordingly, it would appear that the principal role of the marginal *vein* is to function as the *arterial* supply to the developing autopod; clearly this vessel also has important typically venous functions. It appears likely therefore that the cascade of events that normally leads to programmed cell death in this region may have a vascular trigger, and probably requires an adequate blood supply to the target area.

Fewer apoptotic bodies were observed in experimental limbs when the marginal vein was dilated and congested compared to the situation observed in matched control material. If there are triggering "factors" produced by other tissues which are brought to the interdigital zones through the blood supply, then their decrease is also likely to have an inhibitory effect on apoptosis. It has been postulated that reactive oxygen species can be produced by the ischaemia/reperfusion mechanisms after vascular disruption and hypoxia during embryogenesis in the rat (Fantel *et al.,* 1992a; 1992b). It is also relevant in this context that the proto-oncogene, bcl-2, which can block apoptosis is believed to function as an apoptotic inhibitor through its antioxidant pathway (Hockenbery *et al.,* 1993). Alternatively, the original "initiation" message for apoptosis may arise from the apical ectodermal ridge (AER) and, under normal circumstances, be boosted by vascular factor(s) delivered to the site by the marginal vein.

5.4.2 Possible effect of amniotic sac puncture in the inteference of developmental programs in the interdigital zones

Amniotic sac puncture, therefore, for reasons that have yet to be fully established, but probably as a result of the loss of amniotic fluid and a consequent increase in the pressure within the amniotic compartment (see Introduction), results in a transient period of bradycardia lasting about 1.5 - 2h (Chang and Kaufman, 1997). This almost immediately produces venous stasis within the marginal vein of the handplate/footplate and a reduction in the oxygenation of the subjacent tissues. As a consequence of the latter, we believe that the normal sequence of events within the interdigital zone is, at least temporarily, disrupted. This will almost certainly have a long-term effect on the sequential events that lead to the normal development of the hand- and footplate, probably by locally interfering with the normal (i.e. genetic) control of apoptosis in the interdigital zones.

Inhibition of programmed cell death in the interdigital zone was also observed in chick embryos either following the removal of the AER (Hurle and Gañan, 1987) or after the injection of Janus Green B (Fallon 1972; Fernandez-Teran and Hurle, 1984) into the amniotic cavity. In both cases, the epithelial-mesenchymal interaction at the peripheral margin of the limb-buds was interrupted and the apoptotic program in the interdigital zones inhibited. Mesenchymal cells in these regions, instead of dying, differentiated to form

ectopic cartilaginous elements (Hurle and Gañan, 1987). In the mouse mutant *hemimelia-extra toe* (Hm^x), abundant mitotic activity is observed in regions of mesenchyme where extensive evidence of apoptosis is usually seen in the normal limb (Knudsen and Kochhar, 1981). This strongly suggests that the interdigital mesenchymal cells probably possess a number of pre-set programs, so that a certain set of circumstances normally leads to a particular response. Although most cells in this region normally disappear as a consequence of programmed cell death, it is possible that the vascular changes that occur as a consequence of amniotic sac puncture-induced bradycardia may interfere with these apoptotic message(s), and stimulate the interdigital mesenchymal cells into other (aberrant) programs. This is likely to be the explanation for our recent finding of the abnormalities of, but particularly fusion and skeletal "bridges" between, the distal phalanges (acrosyndactyly; see figures 5.2 h and also figures 3.2 d and 3.2 e) in the limbs of experimental fetuses following amniotic sac puncture, in which no other skeletal lesions were observed in the limbs, despite the presence of partial or complete syndactyly (Chang *et al.*, 1996). This probably explains the finding in the (8h + ASP) group in which extensive evidence of mitotic activity was observed around the marginal vein, while the incidence of apoptosis normally observed at this site appeared to be dramatically reduced.

5.4.3 Possible involvement of macrophage-carrying factor(s) in the increased interdigital cell proliferation following amniotic sac puncture

By studying the presence of the antibody (F4/80) produced by monocyte-derived macrophages, Hopkinson-Woolley *et al.* (1994) demonstrated that recruitment of macrophages occurs in the vicinity of dead/necrotic cells which, in their study, increased in number in association with a burn lesion induced on day 12 (E11.5), although an excisional wound was not able to attract macrophages until as late as day 15 (E14.5), presumably due to the greater number of dead/necrotic cells associated with a burn. In our study we observed clusters of dead/necrotic cells in the interdigital zones of the limb bud within 30 minutes after amniotic sac puncture (carried out at 10.00 on day 13), and believe that these would also be expected to attract large numbers of macrophages, as was observed in the burns study (see above). During their clearing of the dead/necrotic cells in the interdigital areas, these macrophages may also bring growth factor(s) to the interdigital zone, and thus stimulate cell proliferation (Chang and Kaufman, 1997). The combination of increased cellular proliferation, and reduced apoptosis inevitably leads to some degree of syndactyly.

In the light of the above discussion, one may raise a question about why the mitotic activity was *decreased* at specific early times after ASP, while it was *increased* at later times after ASP. A possible explanation could be due to an early reduction of the blood supply to these regions, while at later times after ASP the developmental program of apoptosis was deviated towards mitosis.

5.4.4 Increased necrosis as a deviation of developmental programs following amniotic sac puncture

Results in this study appear to be consistent with the view that during the early differentiation of the autopod, a relatively small proportion of the mesenchymal cells in the interdigital zone are involved in cell proliferation, others are removed through apoptosis, while yet others remain either in an undifferentiated state or, under special circumstances, may be capable of differentiating into pre-cartilaginous cells. During the normal development of the autopod, relatively few cells die through the process of necrosis. This situation is in marked contrast to that observed in the experimental limbs where necrotic cells are commonly encountered in the interdigital zones. The most likely explanation for this phenomenon is that these cells die primarily because they receive an inadequate level of oxygenation/nutrition, and that this is as a direct consequence of the transient period of amniotic sac puncture-induced bradycardia (see above) observed in this model which occurs during a critical period of limb/digit morphogenesis. Syndactyly almost certainly results from an alteration in the dynamic equilibrium between these various factors occurring within the interdigital zone.

5.4.5 Comparison of findings in the mouse with those in the rat

Since this is the first report which has examined the activities of sub-populations of cells within the *mouse* autopod following ASP, is is virtually impossible to compare findings in the present study with those of other researchers working in this field.

The experimental findings reported in this study are, in general terms, similar to those reported previously in the rat (Love and Vickers, 1972; Singh and Singh, 1973; Kennedy and Persaud; 1977; Houben, 1984), in that dysmelia following amniotic sac puncture appears to be related to an interruption in, or interference with (often transient), the vascular supply to the autopod, and that as a consequence abnormal patterns of development, such as acrosyndactyly were commonly observed. However, it was particularly surprising that a relatively brief period of vascular disruption following ASP could produce such extensive developmental lesions as reported here. By contrast to the findings reported by Kennedy and Persaud (1977; see also other references cited above) all dead embryos were excluded from the present study. Although this has undoubtedly influenced the severity and incidence of the lesions observed, the exclusion of dead embryos provides a more realistic indication of the incidence of gross limb lesions observed in those fetuses that would have survived to term following ASP than had this material been included in the analysis. Equally, most of the limb lesions observed following ASP are less severe than the most extreme types of limb abnormalities observed clinically following CVS.

At thirty minutes after ASP in rats, ultrastructural examination revealed that although the AER region apparently remained unchanged, intercellular contacts between the ecto-mesodermal interface showed early evidence of disruption; the filopodial connections to the basement membrane became stretched and possibly damaged (Houben and Huygens, 1987), but apparently showed evidence of recovery within about 24 hours.

Although ASP in rats often produces a more severe spectrum of limb defects than observed in our mouse model, it is likely that similar ultrastructural anomalies to those reported in the rat may also be observed in the mouse.

Table 5.1 Total nuclei observed and mitotic indexes calculated from analyses of photomicrographs of interdigital spaces in control and experimental limbs. Three sections from each limb were photographed and printed to a final magnification of x 530. The area of interdigital mesenchymal tissue was measured using a Kontron image analysis system. A transparent overhead, with three areas measuring the equivalent of 50 μm x 50 μm, was randomly placed on the photomicrographs and the total number of intact nuclei within each square was counted. Total number of intact nuclei within each interdigital mesenchymal zone was then derived. Total number of mitotic figures within the interdigital tissue was also counted and mitotic index (per 1000 cells) calculated.

ASP	"internal" control			Experimental		
	total nuclei* (n=limbs analysed)	mitotic figures	mitotic index**	total nuclei* (n=limbs analysed)	mitotic figures	mitotic index**
+ 4 hours	5393 (3)	71	13.16	7185 (4)	29	4.04
+ 8 hours	6653 (3)	57	8.57	8596 (4)	68	7.91
+ 12 hours	7434 (3)	43	5.78	11186 (5)	99	8.95
+ 24 hours	5420 (3)	30	5.54	10301 (5)	99	9.61
+ 36 hours	7615 (4)	18	2.36	9002 (4)	37	6.24

* from 3 histological fields per limb

** per 1000 cells analysed

Table 5.2: Maximum likelihood estimates of mitotic index and chi square test for equality. For easier mathmatic calculation, a figure of estimated mitotic index is derived from each mitotic index presented in Table 5.1. To test whether the mitotic indexes are equal between the "internal" control and experimental groups, the log likelihood ratio test is applied. To fulfill the requirement of this test, a "combined" group, which includes all the data from both the "internal" control and experimental groups, is generated. Following the calculation of the log likelihood ratio of these three groups, a value of χ^2 is derived and the equality (of difference) between the "internal" and experimental groups determined. For detailed statistical methods for evaluation of mitotic indexes and log likelihood ratio test, see the appendix to chapter 5.

ASP + h	"internal" control		Experimental		Combined*		
	mitotic index***	±s.e.m.	mitotic index***	±s.e.m.	mitotic index	±s.e.m.	χ^2
+ 4	13.27	0.7367	3.71	0.2030	8.42	0.3284	252.32 ($p<0.5$)**
+ 8	8.61	0.4847	7.75	0.3364	8.10	0.2934	2.04 ($p>0.5$)
+ 12	5.85	0.3187	8.86	0.3559	7.80	0.3177	35.87 ($p<0.5$)
+ 24	5.39	0.2909	9.85	0.3826	8.33	0.3360	76.34 ($P<0.5$)
+ 36 hrs	2.43	0.1076	4.01	0.1803	3.21	0.0930	62.85 ($p<0.5$)

* see Materials and Methods section for definition.

** The 5% critical value for χ_1^2 is 3.86; therefore all findings significantly different except for those from the ASP+8 hours group.

***These figures differ slightly from the mitotic index figures shown in Table 5.1, because they have been calculated using the approach shown in the appendix to the Materials & Methods section.

Figure 5.1 Representative photomicrographs of interdigital spaces to demonstrate the appearance of mitotic, pyknotic and apoptotic cells on conventional paraffin sections stained with haematoxylin and eosin.

(a) cells in mitosis (arrows). Note condensed chromosomes are observed between the pair of dividing cells

(b) a number of pyknotic cells (arrows). A characteristic condensation of the chromatin into a densely staining mass is noted in these cells.

(c) cells showing the morphological features of apoptosis (arrows). Apoptotic bodies are characterised by the presence of dense, fragmented nuclei, being engulfed by monocyte-derived macrophages.

All photographed under x100 oil immersion objective (final magnification x 1000).

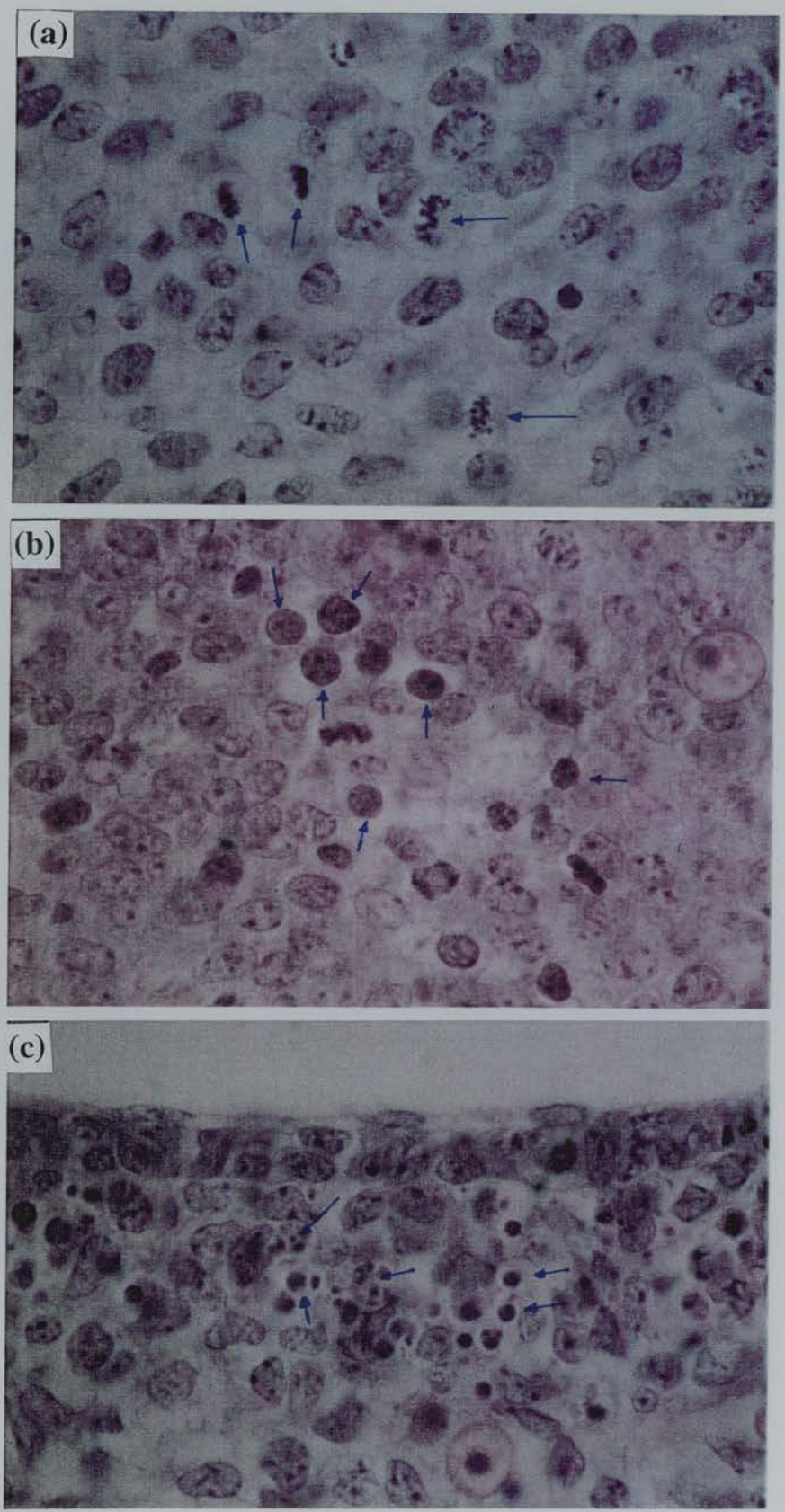

(a)
(b)
(c)

Figure 5.2 Representative coronal histological sections through the forelimbs of "internal" control and experimental embryos previously subjected to amniotic sac puncture (ASP) at various time intervals. Before the specimens were isolated and processed; matched "internal" control limbs were isolated at similar times from the contralateral (non-experimental) uterine horns. Note the interdigital indention of the experimental limbs which are clearly less pronounced than those of the matched control limb (see below). A representative photograph to show extensive haemorrhagic lesions in the experimental limbs isolated later (36h) following ASP. Additional observation of a pre-cartilaginous "bridge" in the experimental hindlimb is also displayed.
All specimens stained with haematoxylin and eosin.

(a) "internal" control limb isolated at 4h after ASP carried out to embryos in contralateral uterine horn. Overview of intact limb.
(b) Overview of intact "internal" control limb isolated at 24h after ASP carried out to embryos in contralateral uterine horn. Note the increasing identation of the interdigital zones compared to the situation illustrated previously in (Figure 5.2a). The marginal vein is no longer evident in control limbs at this time.
(c) Experimental limb isolated 30 min after ASP. Overview of intact limb. Note the interdigital indentation shows an equivalent level to that of the "internal" control limb isolated at the same time (see Figure 5.2a).
(d) Experimental limb isolated 4h after ASP. Overview of intact limb. An equivalent level of interdigital indentation to that of the matched "internal" control limb (data not shown) is observed.
(e) Overview of intact experimental limb isolated at 24h after ASP. Note that the marginal vein is still present, and that the degree of indentation seen in the interdigital spaces is substantially less than that seen in matched control limbs (see Figure 5.2b).
(f) Overview of another intact experimental limb isolated at 24h after ASP. In this example, the gross morphology of the limb bud is abnormal, and there is little evidence of indentations at the peripheral margins of the interdigital spaces.
(g) Overview of intact experimental limb isolated at 36h after ASP. Note extensive areas of haemorrhage (arrow) in the lateral part of this limb, and minimal evidence of indentation in the peripheral parts of the interdigital spaces.
(h) Overview of hindlimb isolated at 36 hours after ASP, showing an example of a pre-cartilaginous "bridge" between two distal phalanges. The latter links the distal phalanges of two of the central digits and spans the intervening interdigital zone. This would have given rise to a form of acrosyndactyly in which the two distal phalanges would have been joined by a skeletal "bridge".
(i) Higher magnification of one part of (h).
Magnifications: a-h x 40, i x 100.

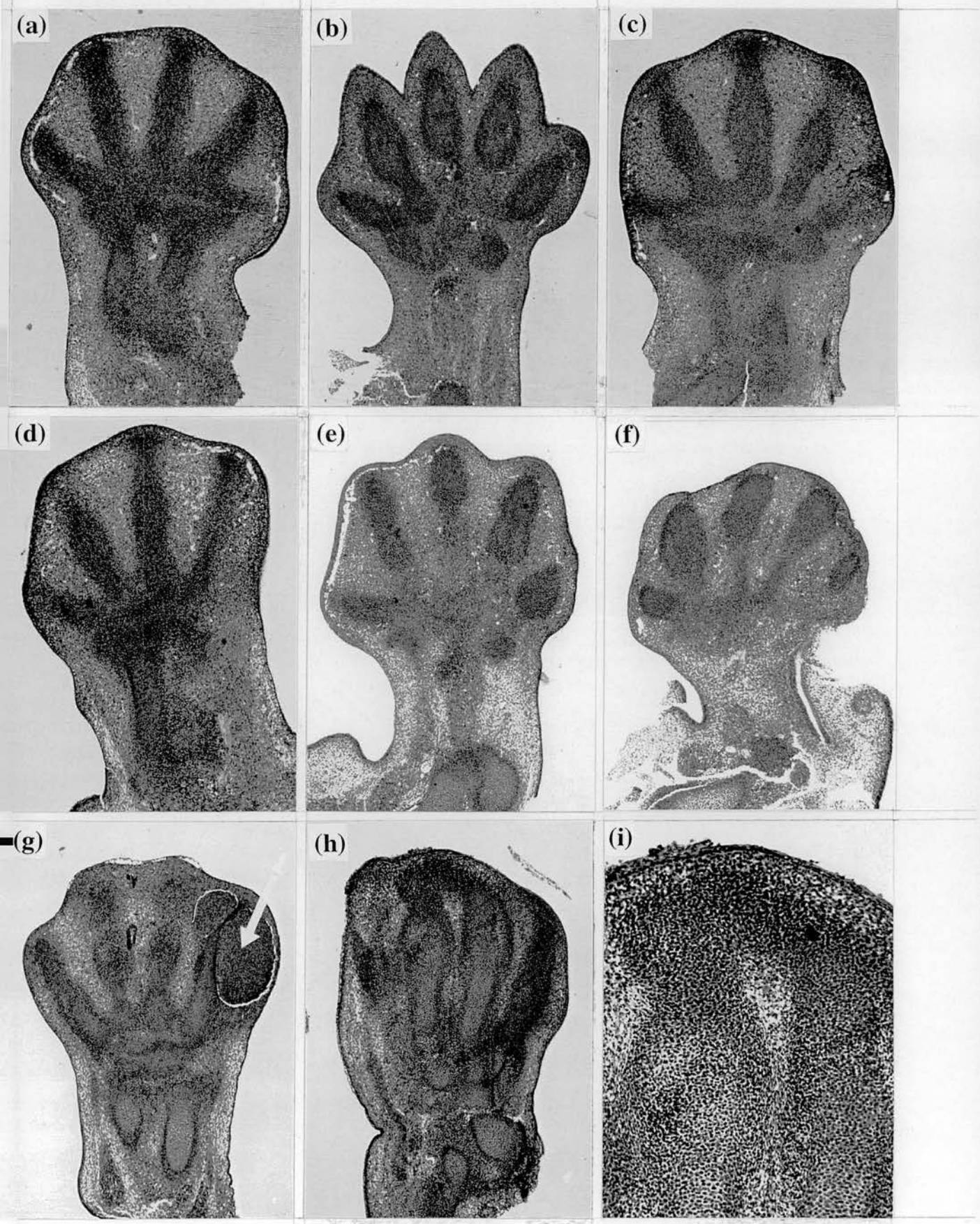

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)

Figure 5.3 Representative photographs to show low and higher magnification views of the marginal vein

of "internal" control and experimental forelimbs following ASP carried out 8h previously. Note the considerable degree of vascular congestion seen in the marginal vein in the experimental but not in the control limb.

(a) low magnification view of the marginal vein of an "internal" control forelimb.

(b) low magnification view of the marginal vein of an experimental forelimb. Clear evidence of vascular congestion is observed.

(c) higher magnification of (a)

(d) higher magnification of (b)

Magnification: a and c, x400; b and d, x1000.

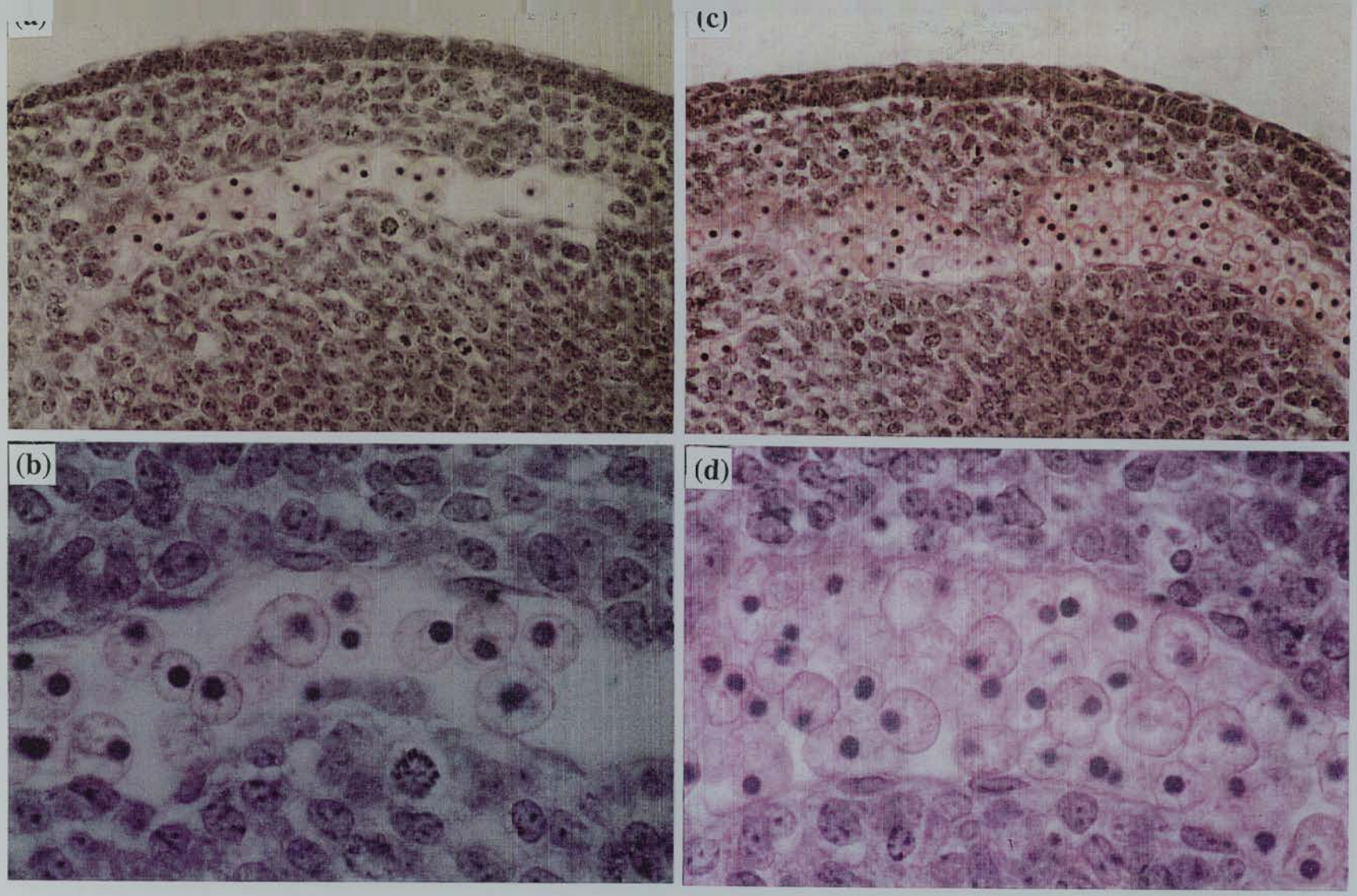

(c)
(b)
(d)

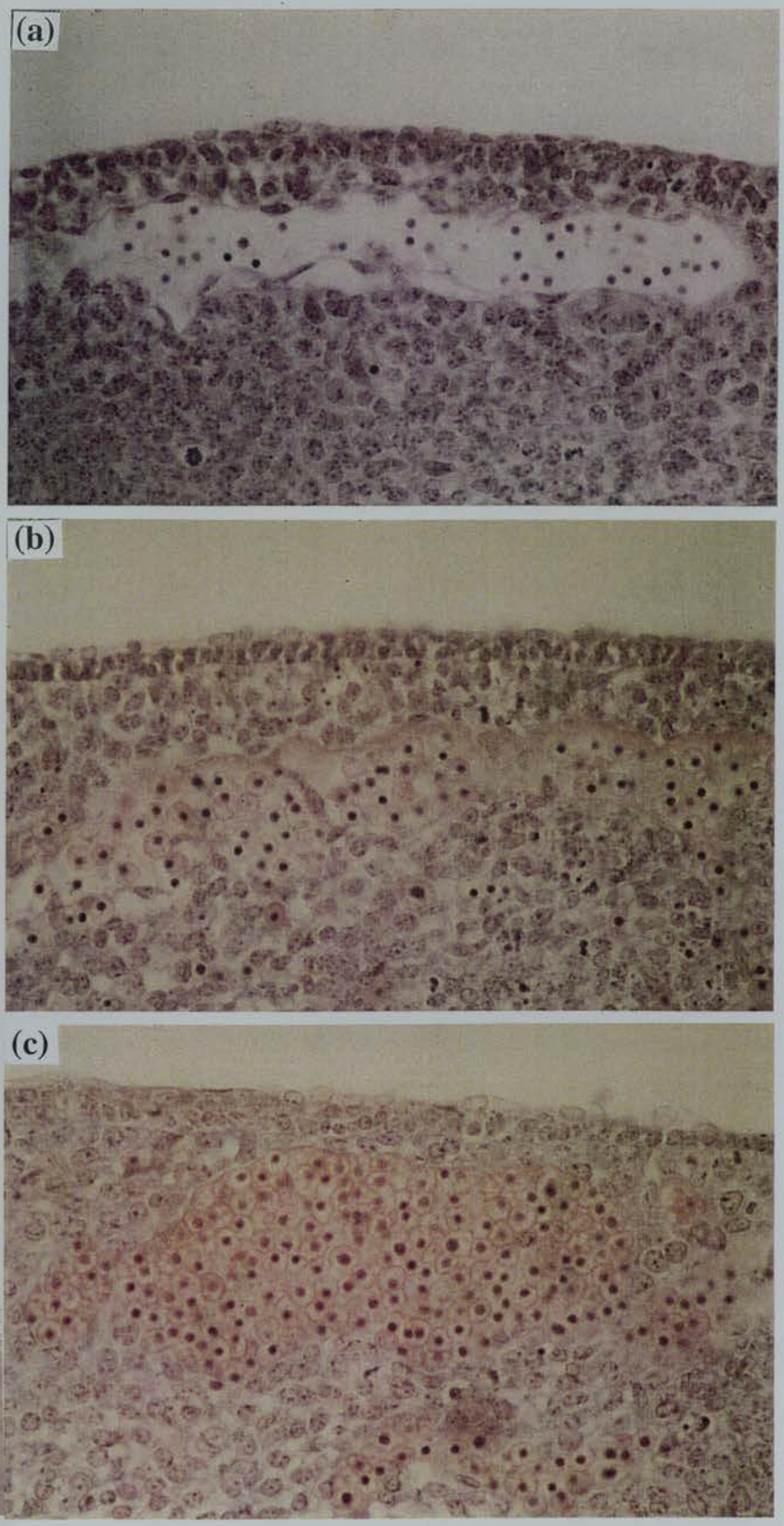
Figure 5.4 Views of the marginal vein of a "non-experimental" control, "internal" control and experimental limb. In the latter two groups, the limbs were isolated at 30 min after the experimental side was subjected to ASP. The "non-experimental" control limb was removed from an embryo that had been isolated from a female that had not been exposed to either an anaesthetic or ASP, at a similar time to those exposed to ASP 30 min previously.

(a) view of the marginal vein of a "non-experimental" control limb to show the normal density of blood cells in this vessel.

(b) view of the marginal vein of an "internal" control limb. Note moderate degree of vascular congestion is shown.

(c) view of the marginal vein of an "experimental" limb. Note extremely severe vascular congestion displayed, possibly involving damage to the endothelial lining of the blood vessel.

Magnification: a-c, x 400


(a)
(b)
(c)

APPENDIX

Statistical methods for evaluation of mitotic indexes

The mitotic index (I) is defined as follows:

$$I = \frac{m \times 1000}{s \times r} \tag{1}$$

where m is mitosis and s a multiplication factor; r is the mean cell count.

This equation is rewritten as follows:

$$r_i = \frac{k_i}{I} \quad \text{where} \quad k_i = \frac{m_i \times 1000}{s_i}. \tag{2}$$

Thus the index is a function of the mean cell counts.

Cell counts are distributed as a Poisson distribution given by

$$Pr[R_i = r_i] = \frac{\mu_i^{r_i} e^{-\mu_i}}{r_i!} \quad \text{where for this case} \quad \mu_i = \frac{k_i}{I}.$$

The best estimate of I is the maximum likelihood estimator $\hat{I}$ which is given by

$$\hat{I} = \frac{\Sigma k_i}{\Sigma r_i}. \tag{3}$$

The standard error (s.e.) of $\hat{I}$ is

$$\text{s.e.}(\hat{I}) = \frac{I^{\frac{3}{2}}}{\sqrt{(\Sigma k_i)}}. \tag{4}$$

To test whether 2 indexes are equal we use the log likelihood ratio test:

$$\begin{aligned}\log \text{lik ratio} = {} & (\Sigma r_{1,i} + \Sigma r_{2,i}) \log \hat{I}_3 - (\Sigma r_{1,i}) \log \hat{I}_1 \\ & - (\Sigma r_{2,i}) \log \hat{I}_2 \end{aligned} \tag{5}$$

where $\hat{I}_3 = \dfrac{\Sigma k_{1,i} + \Sigma k_{2,i}}{\Sigma r_{1,i} + \Sigma r_{2,i}}$

the combined group index derived from (3).

It is known that $2\times$ the log likelihood ratio is distributed as χ^2 with for this case, 1 degree of freedom.

Chapter 6: *Msx-1* alteration and epithelial hypertrophy in the mouse limb following amniotic sac puncture

6.1 Introduction

It is widely accepted that normal digit formation in the chick involves cell death in the interdigital spaces (Saunders and Fallon, 1967). Recently, experimental evidence of programmed cell death (apoptosis) has been shown within the interdigital mesenchyme at gestation stages between day 10.5 and day 15.5 during normal mouse limb development (Zakeri *et al.*, 1994; Mori *et al.*, 1995). If the mesenchymal cells fail to undergo apoptosis, this will result in the absence of discrete digit formation and consequent fusion of digits, a condition termed syndactyly (Menkes and Deleanu, 1964; Hinchliffe and Thorogood, 1974; Pautou, 1976).

Although the induction of programmed cell death within the interdigital mesenchyme is not well understood, experimental data so far available strongly suggest that the interdigital ectodermal tissues may play a crucial part in the process (Kelley, 1973; Hurle and Colvee, 1982). This is based on the different morphology observed in the ectoderm overlying the digital and interdigital zones during the formation of digits in the human (Kelley, 1973), the chick (Hurle and Colvee, 1982) and the rat (Houben and Huygens, 1987). Changes in morphology can be found within the epithelium *per se*, the basal membrane, and in the sites of critical epithelio-mesenchymal interactions. A good example of morphological changes during limb development can be found in the human, where the epithelium is said to be "thinner" in the interdigital regions and "thicker" in the digital regions during Carnegie Stages 17-18 (Kelley, 1973). In the rat, the interdigital and the digital regions are described as being alternately covered with "dense" and "thin" apical ectoderm (Houben and Huygens, 1987). In another study on chick and duck foot development, using scanning electron microscopy, Hurle and Colvee (1982) reported that the epithelium overlying the interdigital epithelium had a more "rounded" appearance and possessed abundant microvilli during digit formation in the chick hindlimb, although similar features were less obvious in the duck hindlimb. Hurle and Colvee (1982) also found three major histological features in the duck interdigital zones: (i), a high level of deposition of collagenous material at the epithelio-mesenchymal interface, (ii) rupture of the basal lamina, and (iii) desquamated ectodermal cells that could be found in the amniotic fluid (Hurle and Colvee, 1982; also Hurle and Fernandez-Teran, 1984). Similar observations were found in the chick, although they were less prominent than those in the duck. These findings suggested that '*the interdigital ectodermal tissue might play a significant role in digit "detachment" from the mesenchymal tissues*' (Hurle and Colvee, 1982).

It was therefore proposed, based on morphological observations, that the necrosis/apoptosis detected within the interdigital zone during digit formation may be related, in some way, to the interaction

between the mesenchymal and the epithelial components (Hurle and Fernandez-Teran, 1984). Histological analysis of chick limb syndactyly induced by Janus Green B revealed that necrotic cells and macrophages were located between the surface ectoderm and its subjacent mesenchyme, which was occasionally accompanied with disintegration of the basal surface of the ectodermal tissue as well as invagination of the ectodermal cells towards the mesenchymal tissue (Fernandez-Teran and Hurle, 1984). In *Xenopus*, however, it has been suggested that digit formation does not necessarily require necrosis within the interdigital zones (Cammeron and Fallon, 1977), which is a feature normally observed in birds, reptiles and mammals (Fernandez-Teran and Hurle, 1984). These findings suggest that there may be, at least in *Xenopus*, a selective pathway for limb morphogenesis other than that observed in birds and mammals. The possibility exists, therefore, that alterations in the overlying epithelium of the interdigital zones or interference with the normal epithelial-mesenchymal interaction could subsequently affect the differentiation of subjacent mesenchymal tissues, leading to the formation of webbing or syndactyly.

In the previous chapters, a mouse model has been established to study the OMLHS (the oromandibular-limb hypogenesis syndrome, Froster-Iskenius and Baird, 1989). A condition similar to the OMLHS was induced in the mouse by amniotic sac puncture (ASP) carried out at the corresponding gestational age when CVS would normally be performed clinically in the human (MacIntyre *et al.*, 1995). Syndactyly/brachysyndactyly/adactyly was found in 74 % of the limbs with morphological abnormalities in the mouse model. Subsequent studies revealed that the condition of syndactyly observed in the model was restricted to the soft tissues in the interdigital spaces (see chapter 3; Chang *et al.*, 1996). Histological analysis in the experimental limbs confirmed that there was evidence of vascular disruption in the marginal vein and in the interdigital capillaries shortly after ASP. Furthermore, it was revealed (in chapter 5) that the dynamic equilibrium of the pre-set programs in the interdigital zones, such as for programmed cell death and cell proliferation were altered, in that the incidence of apoptosis was decreased, while cell proliferation was increased. It was proposed that the alteration in the various pre-set programs in the interdigital zones might result in webbing/syndactyly (see chapter 5; also in Chang *et al.*, 1998).

A number of genes express temporally and spatially in the interdigital zones during digit formation. These include *Msx-1* and *Msx-2* (early stages: Robert *et al.*, 1989; Hill *et al.*, 1989; Davidson *et al.*, 1991; Monaghan *et al.*, 1991; MacKenzie *et al.*, 1992; later stages: Reginelli *et al.*, 1995), *Bmp-2*, *-4*, and -7 (Jones *et al.*, 1991; Francis-West *et al.*, 1995; Lyons *et al.*, 1995), *FGFR 1* and *2* (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1992). It has been proposed that *Msx-1* expression is required to maintain certain cells in a proliferating and undifferentiated state, and that it may also be associated with the limb regeneration in some species (Song *et al.*, 1992; Crew *et al.*, 1995). When an amputation was carried out within the domain of *Msx-1* expression, regeneration of the mouse limb digit tip was observed with re-expression of *Msx-1* in the distal amputated mouse digit in the mesodermal cells of

the blastema-like structure underlying the thickened wound epidermis (Reginelli *et al.*, 1995). These results are consistent with the proposal that *Msx-1* is a candidate gene whose expression in cells at the amputation site is important for the induction of limb regeneration (Reginelli *et al.*, 1995). It is of interest that *Msx-1* expression is also observed in the developing murine molar tooth, and is dependent on a normal reciprocal epithelial-mesenchymal interaction (Jowett *et al.*, 1993; Minkoff, 1991; Kostakopoulou *et al.*, 1996).

When *Msx-1* is abnormally expressed in "determined" myogenic cells, cellular differentiation can be blocked (Song *et al.*, 1992; Wang and Sassoon, 1995). Furthermore, it has been suggested that the proximal limit of *Msx-1* expression defines a boundary zone between the undifferentiated cells and the cells beginning to show signs of differentiation (Wang and Sassoon, 1995). This is of particular interest in our limb model where more than 70 % of the abnormal limbs observed displayed evidence of soft tissue syndactyly, and in which increased mesenchymal cell proliferation has been confirmed (see chapter 5; also in Chang *et al.*, 1998).

In order to evaluate the potential impact of an inadvertent puncture of the amnion during CVS on limb morphogenesis, especially on the "detachment" of the digits, this chapter examined mouse limbs at different time intervals following ASP in semi-thin plastic sections as well as *Msx-1* expression in whole-mounts of limbs following *in situ* hybridization.

6.2 Materials and Methods

6.2.1 mouse (details see section 2.2.1)
6.2.2 procedures of ASP (details see section 2.2.2)
6.2.3 production of "internal" control, "non-experimental" control and "experimental" group (details see section 5.2.2)
6.2.4 Time intervals following amniotic sac puncture (details see section 5.2.4)
6.2.5 Procedures for the semi-thin sections

Females were sacrificed by cervical dislocation at 0.5, 2, 4, 8, 12, 24 and 36 hours following amniotic sac puncture (ASP). Embryos were divided into "internal" controls and the experimental groups based on the operating records. Only living embryos with beating hearts were processed.

Limbs from each time group were isolated from the experimental and the control embryos. All limbs were from embryos that were obtained from several litters. The limbs were fixed in 3 % glutaraldehyde in 0.1 M cacodylate buffer, and retained in this solution for 24 hours. They were then washed in 0.1 M cacodylate buffer with 0.3 % sucrose before a brief period of osmication in 1 % osmium in 0.1M cacodylate buffer. This was followed by two washes in 10 % ethanol. After that,

limb were dehydrated in absolute alcohol. The limbs were finally 'cleared' in propylene oxide before embedding in araldite. Semi-thin (0.75μm) longitudinal (i.e. coronal) sections were cut by a Reichert OMU3 ultramicrotome in order to view both the digits and the interdigital spaces. The sections were mounted on glass slides, stained with toluidine blue, and viewed under a Leitz Laborlux K light microscope.

6.2.6 Wholemount *in situ* hybridization

The technique employed for undertaking non-radioactive *in situ* hybridization for RNA expression in the wholemounts was based on the methodology described by Wilkinson (1993). For the detection of *Msx-1* expression in wholemount preparations of limbs, females were sacrified at 4h, 8h, and 12h after ASP. As described in section 6.2.2, embryos were divided into the "internal" control and the experimental groups. Only limbs from living embryos were processed. All of the control and experimental limbs from the same litter were always processed together in order to compare accurately their staining intensity, since the latter could vary depending on staining time. In all cases, the staining intensity was classed as either weak, medium, or strong, and recorded accordingly. In addition to the "internal" controls and the experimental groups, six litters from the "non-experimental" controls were also retrieved at corresponding gestational stages. The "non-experimental" controls were used to establish *Msx-1* expression during normal limb morphogenesis for comparing with the "experimental" limbs produced in this model. Only left hindlimbs were analysed in this study.

6.3 Results

6.3.1 Light microscopic studies of semi-thin sections

The total number of limbs analysed in this study is listed in Table 6.1. It is clearly apparent that the forelimbs are about 12 hours more advanced in development than the hindlimbs. This is particularly evident in relation to the degree of interdigital indentation (see Figures 6.1 a-b). The abnormalities induced by ASP evidently affected both the forelimb and the hindlimb, although the incidence of abnormalities was slightly higher in the hindlimb due to its earlier stage of development. In both the ASP + 24h and 36h groups, where the morphological abnormalities could be easily confirmed, about 63% of forelimbs and 67% of hindlimbs from the experimental series displayed morphological abnormalities (Table 6.1).

6.3.1.1 Apoptosis

6.3.1.1.1 Control limbs

In the control limbs, apoptotic bodies were clearly observed in the semi-thin sections, usually in the form of multiple residual apoptotic bodies within macrophages (Figure 6.1d). These apoptotic bodies were usually located in sites distal to the marginal vein before evidence of cell death appeared in the interdigital zones. On day 14, clusters of apoptotic bodies were initially found in the interdigital spaces I and IV, and subsequently in spaces II and III. As limb development proceeded, the interdigital spaces gradually became more indented until free digits were observed. Interestingly, during the earlier stages of digit formation, macrophages that contained apoptotic bodies were occasionally observed *within* the marginal vein (Figure 6.1e).

6.3.1.1.2 Experimental limbs

In the experimental limbs, disruption of normal digit formation was often observed. This was particularly seen in the ASP + 24h and ASP + 36h groups. The main features were the reduced incidence of apoptotic bodies in the interdigital zones and the reduced degree of interdigital indentation. In all of the grossly abnormal limbs in these two groups, either none or in most instances very few apoptotic bodies were found in the interdigital zones, which was in marked contrast to the high incidence observed in the control limbs (forelimb: Figure 6.1c; hindlimb: Figure 6.1d). Furthermore, only mimimal evidence of interdigital indentation was observed in these abnormal limbs (forelimb: Figure 6.2 a-b; hindlimb: Figure 6.2 c-d).

6.3.2 Normal and ASP-affected epithelium overlying the interdigital zones

6.3.2.1 Observations of the control series

On day 13 of pregnancy, limbs isolated from the "internal" control group (O + 8h) were analysed. The epithelium overlying both the digital and the interdigital zones consisted of two layers of columnar cells (Figure 6.3 a). By day 14 (O + 24h), shortly after the first evidence of cell death was observed in the subjacent mesenchymal tissues within the interdigital zones, the overlying epithelium exhibited a multiple-layered appearance and appeared to be slightly "thickened" (Figure 6.3b).

6.3.2.2 Observation of the experimental series

In the experimental limbs studied, over 40 % showed evidence of epithelial hypertrophy (Figure 6.3c) which was only observed in two control limbs (one in the day 14, 2pm "non-experimental" control; one in the O + 0.5 "internal" control hindlimb).

Disorganized thickened or unthickened epithelium was also noted in the experimental limbs, especially in the ASP + 12, 24 and 36 hour groups (see Figures 6.2a, 6.3c, 6.4 a-g). This epithelium displayed one or more of the following features: (1) multiple layers of epithelial cells, (2) areas of irregular epithelial distortion, (3) abnormal epithelial desquamation, (4) cavitation within the epithelium, (5) epithelial blebbing, and (6) abnormalities of the basement membrane (see below).

6.3.3 Basement membrane disruption

6.3.3.1 General observations in the control series

During early limb development at the pentagonal stage, the basement membrane was located between the epithelial and mesenchymal cells, running smoothly over the entire surface of the autopod. Shortly later, morphological changes occurred in association with the onset of apoptosis within the interdigital zones. The basement membrane covering the digital areas remained smooth and intact, while that overlying the interdigital areas became more irregular, and zigzagged in appearance (zigzagged; Figure 6.1d). It was unclear, however, whether these changes in the basement membrane resulted in the presence of abundant apoptotic bodies in the mesenchyme, or the alteration within the basement membrane initiated apoptosis. Despite these changes in the basement membrane, epithelial cells did not migrate and became intermixed with the mesenchyme.

6.3.3.2 Basement membrane disruption in the interdigital zone following ASP

Following ASP, the basement membrane exhibited a "ragged" appearance at the early pentagonal stage of limb development, while at later stages, minimal morphological difference was observed between the epithelium overlying the digital zones and that overlying the interdigital zones (see above; photographs of disorganized epithelium associated with basement membrane disruption in Figures: 6.4 b, d, e). This was in marked contrast to the situation observed in the control limbs at the same stage of development (see above; photos of disorganised epithelium in Figures 6.3b, 6.3d, 6.3e).

6.3.4 Vascular events

In both the control and the experimental limbs, spaces were observed between the endothelium and the external wall of capillaries -- a condition termed intra-vascular oedema (also termed *edema venosis*). The intravascular oedema was mainly found in association with the marginal vein. Occasionaly, it was also found in association with the capillaries within the interdigital spaces. In intact vessels, however, no space was observed between the endothelium and the blood vessel wall.

The condition of intra-vascular oedema varied from case to case. In mildly abnormal cases, some degree of separation between the two layers was observed. In severe cases, the two layers appeared to be widely separated. When the severe cases were associated with vascular congestion or haemorrhage, it was often impossible to determine whether the endothelium was intact or damaged. In such cases, the limbs were recorded as having severe congestion rather than evidence of intra-vascular oedema (see table 6.2).

Apart from three hindlimbs in the "non-experimental" series (NX + 3h, NX + 6h, NX + 8h, respectively), which displayed severe evidence of intra-vascular oedema, the rest of the control limbs exhibited either an intact endothelium or displayed mild evidence of this condition (1 in 3 hindlimbs in ASP + 4h, and 1 in 8 forelimbs in O + 12h "internal" control groups). In the experimental limbs, however, mild intra-vascular oedema was found in the majority of limbs in the ASP + 0.5h group, and more than 50 % of the specimens in the ASP + 2 - 24h groups showed severe evidence of intra-vascular oedema.

Severe congestion and/or extensive areas of haemorrhage were also observed either in the marginal vein or in the capillaries within the interdigital spaces in many experimental hindlimbs (1/8 in ASP+2h; 2/3 in ASP+4h; 2/5 in ASP+8h 1/10 in ASP+12h, Figure 6.5).

6.3.5 Cellular changes

6.3.5.1 General observation in the control series

In the control limbs, most mesenchymal cells were polygonal in shape and contained a prominent polygonal nucleus, though in most cells it was difficult to recognize the boundary of the nuclear membrane.

6.3.5.2 Incidence of pyknotic nuclei and other celluar features

In the experimental limbs, three types of cells were recognized within the interdigital spaces: (i) cells which had a similar morphology to those seen in the controls; (ii) cells which possessed a circular (pycnotic) nucleus, i.e. the nuclear membrane was round and smooth with no obvious protrusions, and a clear boundary was present between the nuclear and cytoplasmic membranes, and (iii) an "intermediate" type in which both the nucleus and cytoplasm appeared to be abnormally elongated.

In the experimental series, nuclear features of the pycnotic and the intermediate type were commonly observed between 2 - 12 h following ASP. Interestingly, neither of these nuclear features was observed in the "non-experimental" or "internal" control groups. The distribution of these three catograries of cells in the "experimental" groups is listed in Table 6.3.

6.3.6 Alteration of *Msx-1* expression following ASP

6.3.6.1 *Msx-1* expression during normal limb development

Baseline information on *Msx-1* expression during normal limb development was obtained by *in situ* hybridization on the "non-experimental" controls at intervals at 4, 8, 12, 24, 36 and 48 hours following ASP. On the early afternoon of day 13 (equivalent to ASP + 4 h), *Msx-1* expression was detected around the edge of the pentagonally shaped autopod, and in the interdigital mesenchymal tissues close to the digit boundary. The "internal" controls and the "non-experimental" controls showed comparable results (see Figure 6.6a). Similar results were also found in the limbs isolated at equivalent stages of ASP + 8, 12 and 24h, although the indentations between the digits were deeper than those at +4h after ASP. The interdigital spaces II and III that were eliminated exhibited an apparent diminution in the intensity of *Msx-1* expression (Figure 6.6 b-c). However, *Msx-1* expression was also detected in a narrow area in the peripheral boundary of all of the digits. On day 15, as the activities of apoptosis in the interdigital zones were increased, and individual digits were separated, *Msx-1* expression became gradually restricted to the distal tips of the digits (Figure 6.6c). This was in agreement with the view that *Msx-1* expression at earlier stages matched the areas in the interdigital zones where apoptosis subsequently occurred (early stages: Robert *et al.*, 1989; Hill *et al.*, 1989; Davidson *et al.*, 1991; later stages: Reginelli *et al.*, 1995).

6.3.6.2 Comparison of *Msx-1* expression between the "internal" control and the experimental series

The extent of *Msx-1* expression in both the "internal" control and the experimental limbs is listed in Table 6.4. At 4 hours following ASP (ASP + 4h), the "internal" control limbs showed a considerably lower intensity of *Msx-1* expression when compared with that observed in the experimental limbs (Figure 6.7a). In 6 litters studied, only 3 out of 23 limbs isolated from the "internal" control group showed a moderate degree of *Msx-1* expression. The other 20 limbs just displayed a low level of expression. By contrast, out of 27 experimental limbs, 7 of them exhibited a high level of *Msx-1* expression; 9 of them expressed *Msx-1* at a medium level and only 11 showed a low level of *Msx-1* expression (Figure 6.7a). In those experimental limbs with a high level of *Msx-1* expression, transcripts were seen in the interdigital zones, as well as in areas across the digital boundary. The expression of *Msx-1* even extended towards the zones of digit formation. Similar patterns of *Msx-1* expression were also found in the ASP + 8h group. Approximately 50 % (8 out of 17) of the experimental limbs showed high levels of *Msx-1* expression, while only a small proportion (2 out of 17) of the control limbs exhibited *Msx-1* expression at approximately the same high level.

No difference in *Msx-1* expression was observed between the "internal" control and the experimental groups when they were analysed at ASP + 12h. A low level of *Msx-1* expression was detected in 28 "internal" controls and 21 experimental limbs.

6.3.6.3 *Msx-1* expression in the limbs isolated from dead embryos following ASP

Msx-1 expression was also investigated in the limbs isolated from a number of dead experimental embryos. The results showed that *Msx-1* was only expressed in the middle region of the proximal interdigital zones (Figure 6.7b).

6.4 Discussion

6.4.1 Disruption of the epithelium following ASP

Results in this study clearly demonstrated that the epithelium of the autopod was disrupted following ASP. The disruption of epithelium is presumably due to vascular disruption through the effect of compression from the uterine muscles and the extraembryonic membranes. While the mechanism causing the vascular disruption and the subsequent epithelial disruption is unknown, at least two hypotheses may be proposed based on previous analyses of the ASP mouse model.

Vascular disruption may initially deprive the epithelial and mesenchymal cells of their nutritional supply, leading to a detrimental effect on limb morphogenesis. As a normal blood supply returns, compensatory mechanisms may be triggered, leading to the epithelial hypertrophy seen in this model. Alternatively, the well-balanced epithelial-mesenchymal or intercellular interaction in the limb mesenchyme may be directly or indirectly disturbed by the temporary shortage of blood supply, which in turn causes a hypertrophic effect on the epithelium. These speculations remain, of course, to be supported by experimental evidence.

6.4.2 *Msx-1* expression and epithelial-mesenchymal interaction

It has been hypothesized that a normal epithelial-mesenchymal interaction plays an essential role in the regulation of *Msx-1* expression in the developing molar teeth (Jowett *et al.,* 1993), in the eye (Monaghan *et al.,* 1991), in the mouse limb (Hill *et al.,* 1989; Robert *et al.,* 1989) and in the morphogenesis of the chick limb (Coelho *et al.,* 1991; Robert *et al.,* 1991). Furthermore, a large amount of data derived from tissue grafting and histospecific recombination experiments have shown that the *Msx-1* expression may be altered in many tissues (limb bud: Davidson *et al.,* 1991; Robert *et al.,* 1991; Haramis *et al.,* 1995; Kostakopoulou *et al.,* 1996, facial processes: Takahashi *et al.,* , 1991; Brown *et al.,* 1993, tooth buds: Jowett *et al.,* 1993, the epithelium of the perinatal Müllerian duct: Pavlova *et al.,* 1994, and also see review by Davidson 1995). In these studies, the level of *Msx-1* expression depended largely on the presence of appropriate epithelial-mesenchymal interaction. When the epithelial-mesenchymal interaction was disrupted, *Msx-1* expression was either altered or absent. Furthermore, Wang and Sassoon (1995) showed in an *in vitro* study that disruption of cell-cell interactions in the limb mesenchyme resulted in a substantial decrease in *Msx-1* expression. In our studies we found that the normal level of *Msx-1* expression, which was present in the interdigital zones of the developing autopod, was elevated (i.e. there was over-expresssion) following ASP. The reason for this finding is unclear.

One possible explanation for the increase of *Msx-1* expression is that, although the normal epithelial-mesenchymal interaction has clearly been modified, a relationship between these two tissues still exists, which is unlike the situations in the tissue grafting and recombination experiments where such a relationship was often completely disrupted. Another possibility may be that when the vascular disruption occurred following ASP, some unknown factors, for example cytokines, might have been released as a result of haemorrhage and other forms of vascular disturbance, leading to subsequent involvement in triggering the over-expression of *Msx-1*. One candidate factor for the increase of *Msx-1* expression is Bmp-4 protein which normally precedes *Msx-1* expression during tooth morphogenesis (Vainio *et al.,* 1993).

6.4.3 *Msx-1* and apoptosis

Both *Msx-1*and *Msx-2* genes are believed to be associated with programmed cell death (apoptosis) in the interdigital areas of the limbs. This is on the basis that their expression correlates temporally and spatially with areas of apoptosis in these regions (Robert *et al.,* 1989; Hill *et al.,* 1989; reviewed by Davidson, 1995; for correlation at later stages see Reginelli *et al.,* 1995). This, however, contradicts the results in this study. It is expected that *Msx-1* expression would be either inhibited or at the least down-regulated in the interdigital zones, since apoptosis had largely been abolished in these regions. Instead of inhibition or down-regulation, however, *up-regulation* of *Msx-1* expression was observed in the interdigital zones.

One possible explanation for this contradiction may be similar to that suggested in a report regarding *Msx-1* knockout mice (Satokata and Maas, 1994). Although it had been suspected that *Msx-1* might play a key role during limb morphogenesis, no obvious phenotypic abnormalities were found in the limb of the *Msx-1* knockout mice. *Msx-1* may therefore be compensated by *Msx-2* during normal limb development (Satokata and Maas, 1994; also see Reginelli *et al.,* 1995). Another possibility is that although *Msx*-1 (and also *Msx-2*) expression is detected in areas *preceding* apoptosis (Hill *et al.,* 1989; Robert *et al.,* 1989; Reginelli *et al.,* 1995), no *direct* evidence so far has been available to unequivocally establish a direct link between *Msx-1* and apoptosis. Apart from that observed in the polydactylous chick (Coelho *et al.,* 1993) and from the retinoic acid studies in the same report, programmed cell death was inhibited when corresponding expression of *Ghox-7.1* declined (Coelho *et al.,* 1993). In chick grafting experiments involving the facial processes, it was claimed that *Msx-1,* instead of *Msx-2,* had a "less pronounced" relationship with regard to apoptosis (Brown *et al.,* 1993). While the expression of *Msx-2* was demonstrated to precede and directly induce apoptosis in the chick rhombencephalic neural crest (Graham *et al.,* 1993; 1994) and in *Msx-2* transfected myogenic cell lines (Marazzi *et al.,* 1997), the relationship between *Msx-1* and apoptosis remains unresolved. There remains another possibility that, if *Msx-1* is involved as a factor prior to apoptosis, an appropriate amount of its transcripts may be needed.

6.4.4 *Msx-1* and cell proliferation

It has also been suggested that *Msx-1* may also be involved in the maintenance of mesenchyme cells in a proliferative and undifferentiated state in the progress zone during limb morphogenesis (Song *et al.,* 1992; Muneoka and Sassoon, 1992). In the myoblast cell lines transfected with *Msx-1*-expressing retrovirus, a more direct relationship was observed, where constitutive expression of *Msx-1* inhibited terminal differentiation. The transfected cells maintained a hyperchromatic state before they were induced for transformation (Song *et al.,* 1992). In previous chapters, an increase of mitotic activity in

the interdigital zones between ASP + 4h to ASP + 12h was found. This result confirmed that, as had been suggested by others (see above), *Msx-1* may play an important role in maintaining the proliferative status of mesenchmal cells. While others had confirmed this relationship in the progress zone of the limb bud at earlier stages of development, this study demonstrated that a similar relationship was also present in the interdigital zones at later stages of limb development.

6.4.5 *Msx-1* and limb regeneration

Msx-1 has been clearly shown to be intimately involved with tissues undergoing regeneration (Crews *et al.*, 1995; Reginelli *et al.*, 1995). Reginelli and co-workers (1995) artificially amputated part of the limb bud and detected a high level of *Msx-1* expression subjacent to the thickened epidermis in the wound site, where tissue regeneration was found. In contrast, no *Msx-1* transcripts were found in the adjacent areas where no tissue regeneration occurred. Although Reginelli *et al.* (1995) did not describe "hypertrophy" in their report, a thickened multiple-layered and hypertrophied epidermis was clearly illustrated in the figures in their publication. Two common features have therefore been observed in both their study and the present study: (1) the appearance of an irregular-shaped hypertrophied epithelium (or epidermis), (2) a high level of *Msx-1* expression in the hypertrophied region, although the methods used to induce limb abnormalities were obviously different in the two studies.

Despite the fact that no typical features of tissue regeneration were observed in this study, increased mitotic activity and the formation of a skeletal "bridge" were observed between digits in some of the abnormal limbs. This might provide indirect evidence that an occasional response of tissue regeneration could be stimulated in this model. After a prolonged period of bradycardia following ASP, the vascular disruption was gradually eliminated, leaving an *incomplete* regenerative response.

6.4.6 Conclusion

It has clearly been demonstrated in this study that amniotic sac puncture dramatically affects the limb epithelium, probably through altering the local environment in these areas. This inevitably interferes with the epithelial-mesenchymal interaction that would otherwise normally occur in this location. The possibility exists that this might defer the normal timing of programmed cell death that occurs in the interdigital spaces, eventually leading to soft tissue syndactyly as observed in a high proportion of the affected limbs in our Avertin studies.

Table 6.1 Total number of fore- and hindlimbs analysed using semi-thin sections in the "non-experimental"control, "internal" control and experimental groups at intervals following ASP. Number of morphological normal and abnormal limbs in ASP + 24h and ASP + 36h groups is also indicated.

	FORELIMBS				**HINDLIMBS**		
TIME/GROUP	***"non-exp" control***	***"internal" control***	***experimental***	**TIME/GROUP**	***"non-exp" control***	***"internal" control***	***experimental***
ASP + 0.5 h		8	5	ASP + 0.5 h		5	4
ASP + 2 h		7	4	ASP + 2 h		5	8
NX + 3 h	1			NX + 3 h	2		
ASP + 4 h		1	3	ASP + 4 h		3	3
NX + 6 h	1			NX + 6 h	1		
ASP + 8 h	1	3	3	ASP + 8 h	1	3	5
ASP + 12 h	3	8	8	ASP + 12 h	2	10	10
ASP + 24 h	1	2 (2 N + 0 AB)	5 (1 N* + 4 AB#)	ASP + 24 h	1	3 (3 N + 0 AB)	5 (2 N + 3 AB)
NX + 28;32 h	2			NX + 28 h	1		
ASP + 36 h	1	8 (8 N + 0 AB)	3 (2 N + 1 AB)	ASP + 36 h	1	6 (6 N + 0 AB)	4 (1 N + 3 AB)

NX + h : equivalent time when the "non-experimental" control limbs were isolated
N*:Normal morphology
AB#: Abnormal morphology

Table 6.2 Incidence and severity of *edema venosis* of the control and experimental limbs at intervals following ASP. A condition when a space is found between the endothelium and the external wall of capillaries is termed *edema venosis* (also termed intra-vascular oedema). The severity of oedma venosis is classified into three levels, depending on the separation between the endothelium and the external wall of capillaries. In the case of severe vascular congestion, no *edema venosis* was classified.

FORELIMBS

TIME\GROUP	"non-exp" control				"internal" control				experimental				
	t	◊	θ	♦	t	◊	θ	♦	t	◊	θ	♦	CG
ASP + 0.5 h					8	8	0	0	3	3	2	0	
ASP + 2 h					6	3	3	0	4	0	0	4	
NX + 3 h	1	0	1	0									
ASP + 4 h					1	0	1	0	3	2	1	0	
NX + 6 h	1	1	0	0									
ASP + 8 h	1	1	0	0	3	0	0	3	3	3	0	0	
ASP + 12 h	3	3	0	0	8	5	3	0	8	3	3	2	
ASP + 24 h	1	1	0	0	2	1	0	1	5	2	1	2	
NX + 28;32 h	2	2	0	0									
ASP + 36 h	1	1	0	0	8	8	0	0	3	3	0	0	

HINDLIMBS

TIME\GROUP	"non-exp" control				"internal" control				experimental				
	t	◊	θ	♦	t	◊	θ	♦	t	◊	θ	♦	CG
ASP + 0.5 h					5	3	2	0	3	0	2	1	
ASP + 2 h					5	0	5	0	8	0	1	6	1
NX + 3 h	2	0	1	1									
ASP + 4 h					3	1	0	2	2	0	0	0	2
NX + 6 h	1	0	0	1									
ASP + 8 h	1	0	0	1	2	0	2	0	5	0	1	2	2
ASP + 12 h	2	2	0	0	10	6	4	0	8	2	5	0	1
ASP + 24 h	1	1	0	0	3	3	0	0	5	3	1	1	
NX + 28 h	1	1	0	0									
ASP + 36 h	1	1	0	0	6	6	0	0	4	4	0	0	

NX + h : equivalent time when the "non-experimental" control limbs were isolated
t : total number;
◊ : intact endothelium of blood vessel within the autopod
θ : mild edema venosis of the blood vessel within the autopod.
♦: severe edema venosis of the blood vessel within the autopod.
CG: vascular congestion; no edema venosis was classified in this situation.

Table 6. 3 The proportion of limbs displaying pyknotic* (or to be pyknotic#) cells at intervals following ASP. Three classifications of cellular morphology are observed, namely healthy, pyknotic and an intermediate class. Ordinary interdigital mesenchymal tissue are polygonal in shape and contain a prominent polygonal nucleus. Following ASP, a considerable number of limbs display pyknotic cells and cells with the "intermediate" cell type between healthy and pyknotic.

ASP	**Forelimbs**		**Hindlimbs**	
	pyknotic	***intermediate***	***pyknotic***	***intermediate***
+2 hours	25%	not found	not found	25%
+4 hours	67%	not found	100%	100%
+8 hours	33%	33%	40%	40%
+12 hours	not found	50%	10%	40%

* pyknotic cells: cells which possessed a circular nucleus
# cells to be pyknotic: an "intermediate" type of cells in which both the nucleus and cytoplasm appeared to be abnormally elongated. This is a group intermediate beween morphologically normal cells and pyknotic cells.

Table 6.4 Comparison of the *msx-1* expression between "internal" control and "experimental" limbs at intervals following ASP. Samples from opposite horns within the same litter were processed simultaneously in order to accurately reveal the staining intensity (see section 6.2.3).

ASP + 4h	**"internal" CONTROLS**							**EXPERIMENTALS**						
Intensity*	Litter 1	Litter 2	Litter 3	Litter 4	Litter 5	Litter 6	Total	Litter 1	Litter 2	Litter 3	Litter 4	Litter 5	Litter 6	Total
+	4	5	0	5	3	3	20	0	3	1	5	2	0	11
++	0	0	0	0	0	3	3	2	1	1	0	3	2	9
+++	0	0	0	0	0	0	0	3	0	0	0	0	4	7

ASP + 8h	**"internal" CONTROLS**						**EXPERIMENTALS**						
Intensity*	Litter 1	Litter 2	Litter 3	Litter 4	Litter 5	Total	Litter 1	Litter 2	Litter 3	Litter 4	Litter 5		Total
+	1	2	3	2	5	13	1	0	0	0	3		4
++		2				2	1	2	0	0	1		5
+++		2				2	3	0	4	1	0		8

ASP + 12h	**"internal" CONTROLS**							**EXPERIMENTALS**						
Intensity*	Litter 1	Litter 2	Litter 3	Litter 4	Litter 5	Litter 6	Total	Litter 1	Litter 2	Litter 3	Litter 4	Litter 5	Litter 6	Total
+	6	4	4	5	4	5	28	3	3	4	4	3	4	21
++	0	0	0	0	0	0	0	0	0	0	0	0	0	0
+++	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* intensity of staining of limbs within each litter was scored individually and divided into three levels.

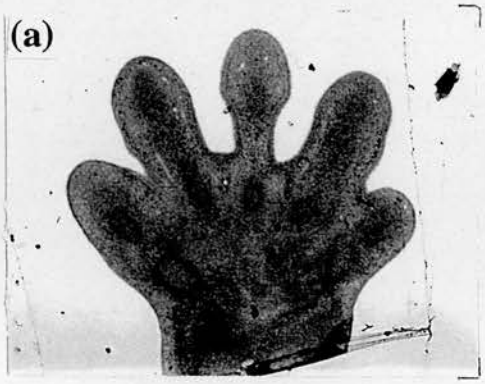
Table 6.5 *Msx*-1 expression of "non-experimental" control limbs detected using whole-mount in situ hybridization.
Their equivelent developmental stages to those of the "internal" control and "experimental" limbs following ASP are shown.

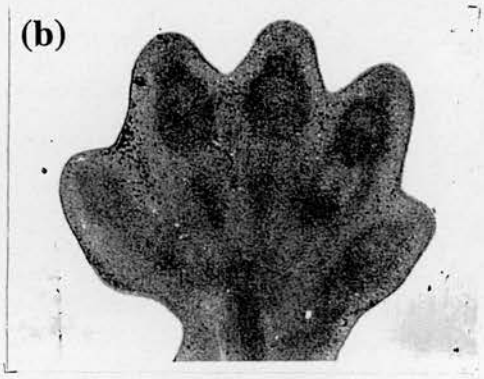
gestational stage	time	ASP + hrs (approximal equivalent)
d13	2.30 pm	+ 4 hrs
d13	6.00 pm	+ 8 hrs
d13	10.00 pm	+ 12 hrs
d14	10.00 am	+ 24 hrs
d14	10.00 pm	+ 36 hrs
d15	10.00 am	+ 48 hrs

A whole litter of each stage was analysed.

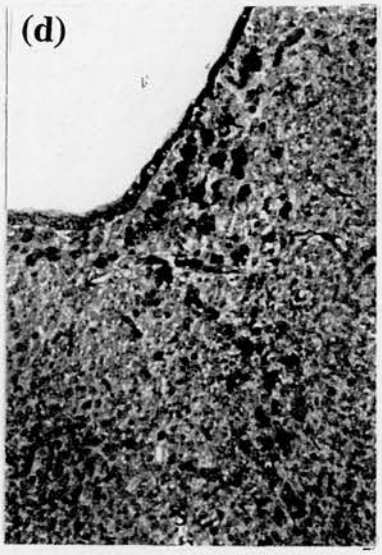
Figure 6.1 Representative photomicrographs of semi-thin sections to show the different developmental stage between the fore- and hindlimb. Interdigital indentation and its relationship with programmed cell death (apoptosis) in the interdigital zones is also displayed. One additional photograph demonstrates the presence of an apoptotic body within the marginal vein.

(a) overview of an "internal" control forelimb isolated 36 hours following ASP (O + 36h). Note the digits are almost separated.
(b) overview of an "internal" control hindlimb isolate 36 hours following ASP (O + 36h). Separation of the digits is less well advanced compared to that observed in the forelimb isolated at the same gestational stage, indicating less advanced development in the hindlimb.
(c) higher magnification of (Figure 6.1a) to show the interdigital indentation between the 1st and 2nd digits. Note only few apoptotic bodies are present along the peripheral boundary of these two digits. The epithelium along the boundary in this area displays a smooth, even-thickness.
(d) higher magnification of (Figure 6.1b) to demonstrate lesser degree of interdigital indentation occurring in the hindlimb compared to that in the forelimb isolated at the same gestational stage. Clusters of apoptotic bodies are observed in this area. The appearance of the epithelium covering the interdigital indentation is irregular and "zigzag".
(e) view of the marginal vein in an "internal" control forelimb isolated 0.5h following ASP (O + 0.5h). Note a macrophage containing apoptotic bodies is present in the blood vessel.
Magnification: (a) and (b), x40; (c) and (d), x250; (e), x400.


(a)


(b)


(c)


(d)

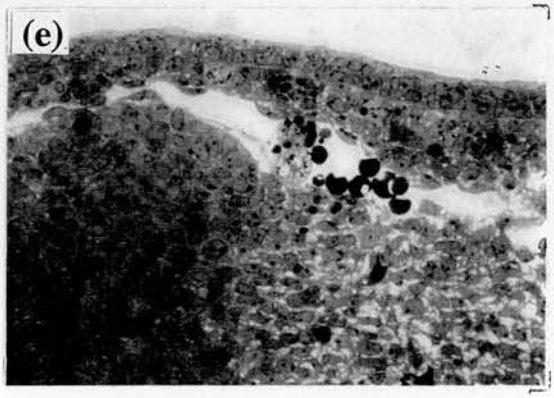

(e)

Figure 6.2 Representative microphographs of both fore- and hindlimbs in the experimental (ASP + 36h) group. Abnormal morphology is observed which associated with less than normal pattern of indented interdigital zones.

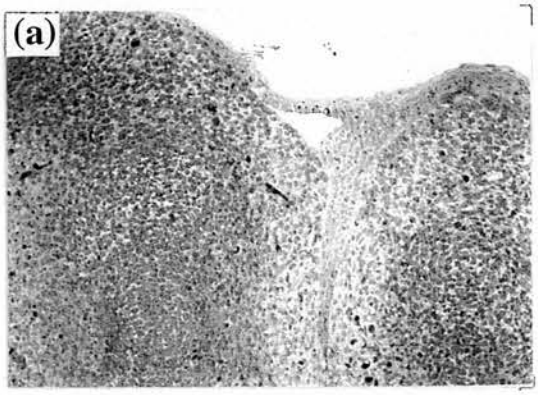
(a) overview of a forelimb isolated 36 hours following ASP. No separation of the adjacent two digits is evident, i.e. very little indentation is observed. Note a combination of detached and extended epithelium is shown overlying the interdigital zone.

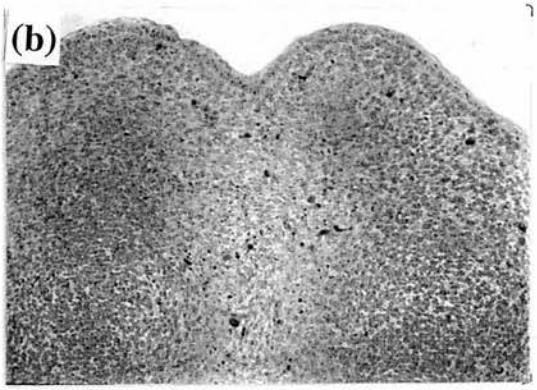
(b) overview of a forelimb isolated 36 hours following ASP. Only very little indentation in this interdigital area is observed. A slightly thinner epithelium (compared to that in Figure 6.2b) is present overlying the interdigital zone.

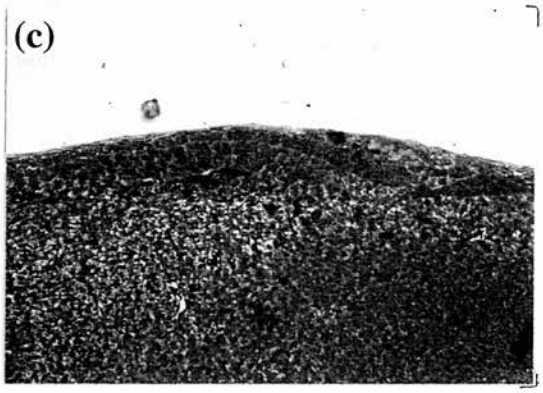
(c) overview of a hindlimb isolated 36 hours following ASP. Note that there is *no* indentation in this particular interdigital space. The epithelium is also slightly disorganized.

(d) overview of a hindlimb isolated 36 hours following ASP. Only a very insignificant degree of indentation of the interdigital zone is present. The epithelium is also slightly disorganized.

Magnification: (a)-(d), x160


(a)


(b)


(c)

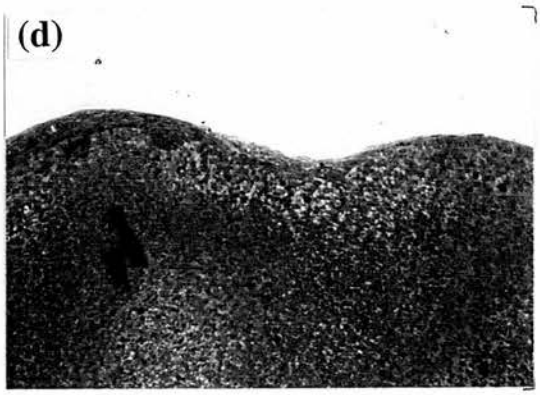

(d)

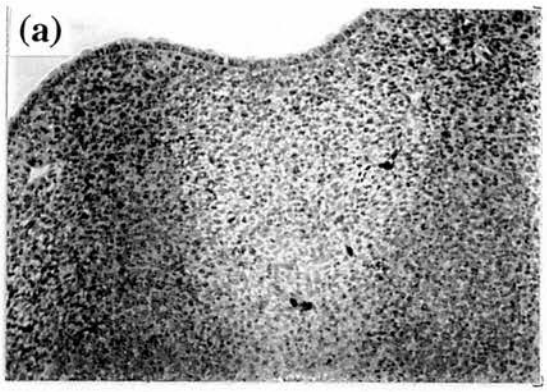
Figure 6.3 Representative photomicrographs to show the different appearance of the epithelium overlying the interdigital zone of the "internal" control and experimental forelimbs.

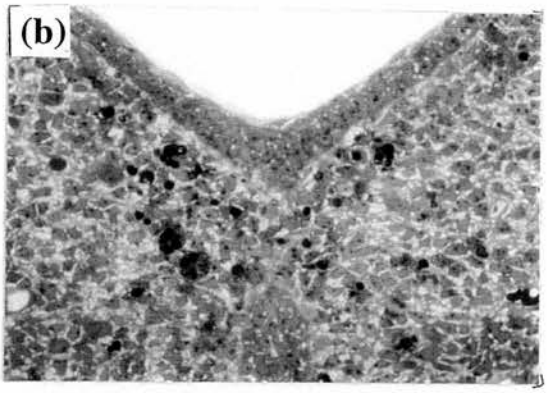
(a) overview of a representative "internal" control forelimb isolated 8 hours following ASP (O + 8h). The developmental stage is equivalent to day 13 of gestation. Note that the epithelium consists of two layers of columnar cells overlying both the peripharal boundary of the digital and interdigital zones of the autopod. A smooth, even-thickened appearance is observed. Desquamated cells spread nicely and evenly along the boundary, without any evident degree of separation from the epithlium.

(b) overview of a representative "internal" control forelimb isolated 24 hours following ASP (O + 24h). The developmental stage is equivalent to day 14 of gestation. The epithelium overlying the interdigital zone consists of multiple layers of cells. At this stage of development, the appearance of the epithelium becomes wedged and slightly "thickened". Similar to the appearance observed in Figure 6.2a, the desquamated cells spread nicely and evenly along the boundary, without separation from the subjacent epithlium.

(c) overview of a representative experimental forelimb isolated 2 hours following ASP (ASP + 2h). An unusual hypertrophied epithelium overlying the interdigital zone is observed. This is a wedge-shaped, multiple-layered structure, with an additional layer of detached desquamated cells towards the interdigital space.

Magnification: (a), x 250; (b) and (c), x400


(a)


(b)

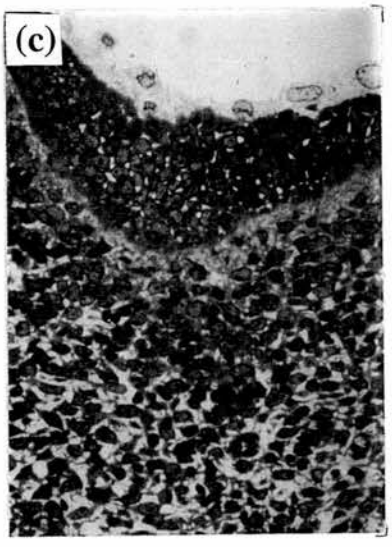

(c)

Figure 6.4 Variety of disorganized epithelial tissues overlying the interdigital zone of experimental limbs at intervals following ASP.

(a) overview of epithelium overlying the interdigital zone of a forelimb isolated 12 hours following ASP. Distorted epithelium is shown.

(b) overview of epithelium overlying the interdigital zone of a forelimb isolated 12 hours following ASP. "Thickened" and uneven appearance of the epithelium is observed. An outer layer of desquamated cells is also seen. Note that the normal contact (basement lamina) between the epithelium and the mesenchyme is distorted.

(c) overview of the epithelium overlying the interdigital zone of a forelimb isolated 24 hours following ASP. Although the appearance of the epithelium is very similar to that observed in the "internal" control limbs, a layer of condensed desquamated cells covering the epithelium is seen.

(d) overview of the epithelium overlying the interdigital zone of a forelimb isolated 24 hours following ASP. Extremely disorganized epithelium with a ragged surface and unexpected cavitation is present. Also note that the contact (basement lamina) between the epithelium and the mesenchyme is ragged.

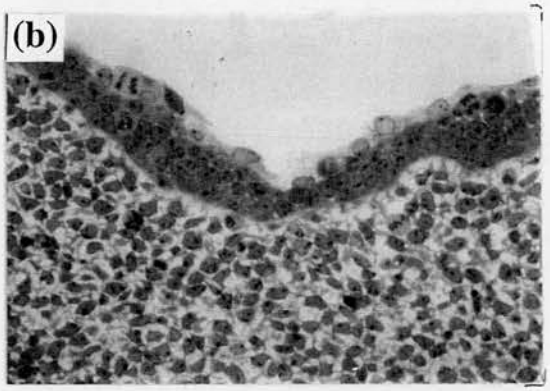
(e) overview of the epithelium overlying the interdigital zone of a hindlimb isolated 12 hours following ASP. Cells within the epithelium are disorientated. The contact line (basement membrane) between the epithelium and the mesenchyme is not smooth as is observed in the "internal" control limb (Figure 6.2a).

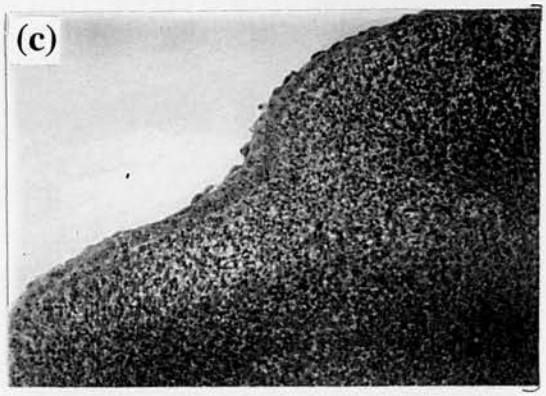
(f) overview of the epithelium overlying the interdigital zone of a hindlimb isolated 24 hours following ASP. Detached epithelium and subsequently formed cavitation between the epithelium and the mesenchyme is shown. Cluster of desquamated cells are located in the area where two parts of the epithelium are in contact.

(g) overview of the epithelium overlying the interdigital zone of a hindlimb isolated 24 hours following ASP. A large cavity is found between the epithelium and the mesenchyme. Hypertrophy of the epithelium is noted in part of the epithelium. Clusters of desquamated cells are noted that are attached to the hypertrophied epithelium. In the middle of the interdigital space, a region of epithelial cells is present.

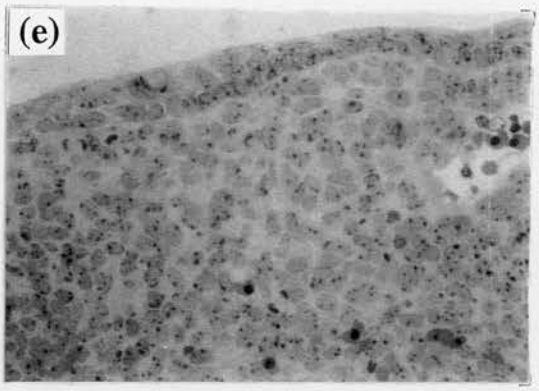
Magnification: (a), (c), (d), (f), and (g), x160; (b) and (e), x400

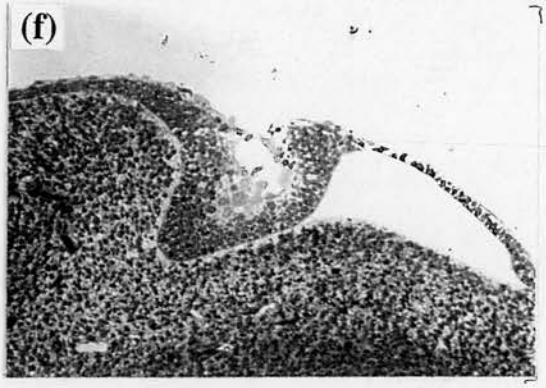

(a)


(b)


(c)


(d)


(e)


(f)

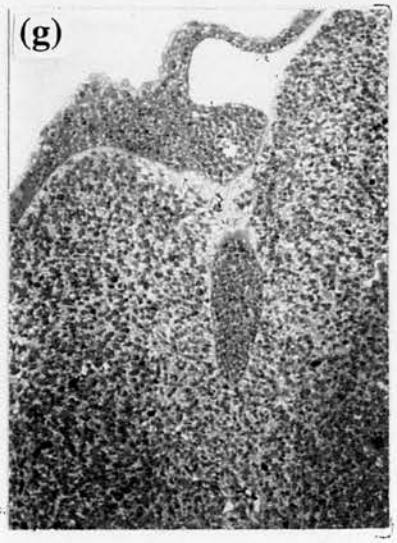

(g)

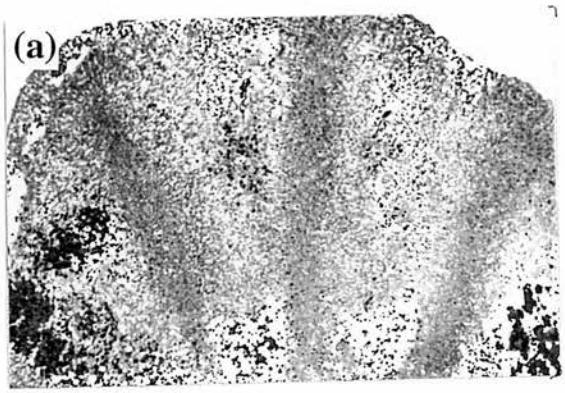
Figure 6.5 Representative photographs to demonstrate typical extensive haemorrhagic lesions often observed in experimental limbs at intervals following the ASP.

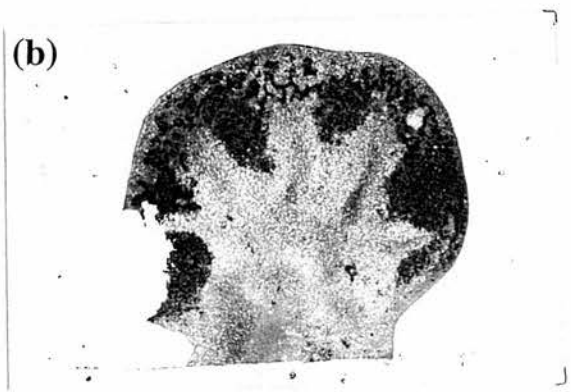
(a) overview of an experimental forelimb isolated in the (ASP + 12h) group. Two large areas of haemorrhage are displaying in the I and IV interdigital zones, respectively. A small patch of haemorrhage is found in the II interdigital zone. No pre-cartiligenous cells appear to be affected by the vascular disruption.

(b) overview of an experimental hindlimb isolated in the (ASP +8h) group. Extensive haemorrhage is encountered in all of the interdigital zones. Distal parts of the digital rays are also affected.

(c) higher magnification of the same experimental limb as shown in Figure 6.5b.

Magnification: (a) x100; (b) x40; (c) x160


(a)


(b)

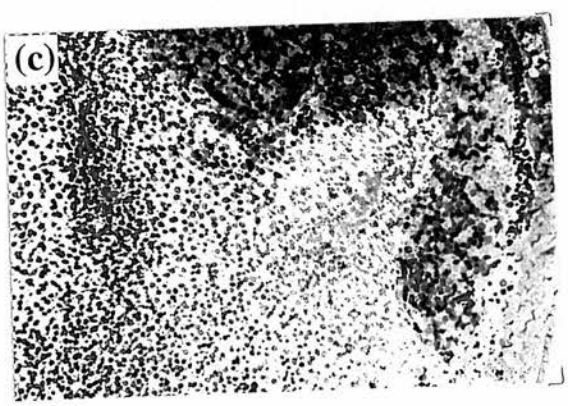
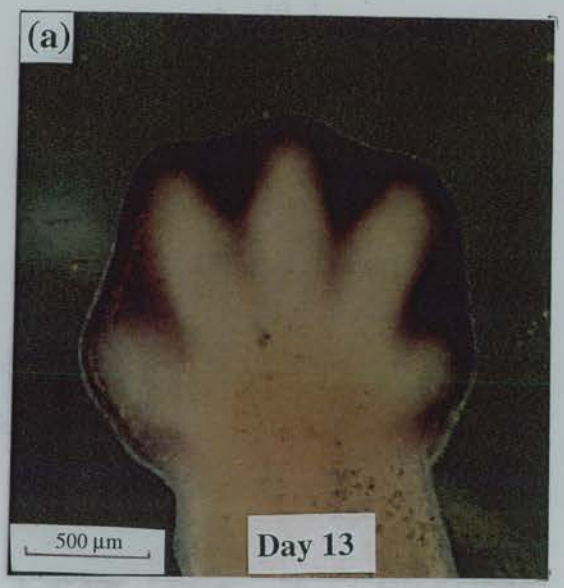

(c)

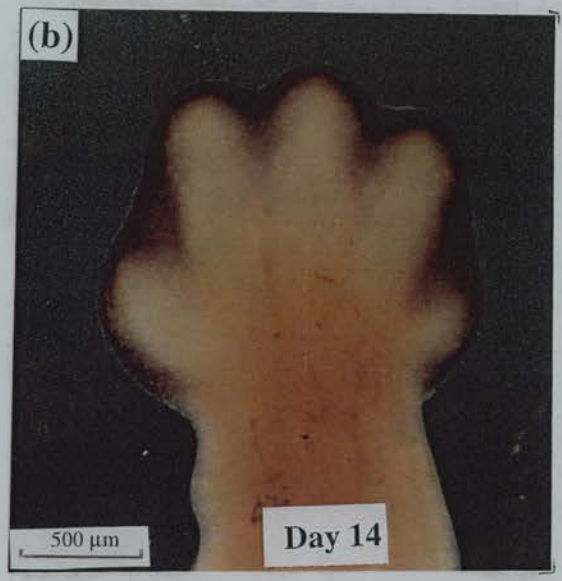
Figure 6.6 Representative photographs showing the expression pattern of *msx-1* gene using whole mount in situ hybridization during normal hindlimb morphogenesis. Hindlimbs isolated from control females at equivalent times to (ASP + 4,8,12,24,36 and 48h) are analysed in this study.

(a) the expression pattern of *msx-1* gene in the limb isolated at 2 pm, day 13 of pregnancy (equivalent to ASP + 4h). A wedge-shaped region of expression is noted in each interdigital zone. Very little indentation in the interdigital zones is observed.

(b) the expression pattern of *msx-1* gene in the limb isolated at 10 am on day 14 of pregnancy (equivalent to ASP + 24h). Lesser areas of *msx-1* expression are displayed, possibly due to the deeper indentation of the interdigital zones.

(c) overview of the expression pattern of *msx-1* gene in the sequence of limb development. Left: a limb isolated on the morning of day 13 of gestation, showing a similar result to that shown in Figure 6.6a. Middle: a limb isolated on the morning of day 14 of pregnancy. Similar expression pattern to that shown in Figure 6.6b is observed. Right: a limb isolated on the morning of day 15 of gestation. Separation between the digits is completed. The expression of *msx-1* is restricted to the very distal end of each digit.


(a)
500 µm
Day 13


(b)
500 µm
Day 14

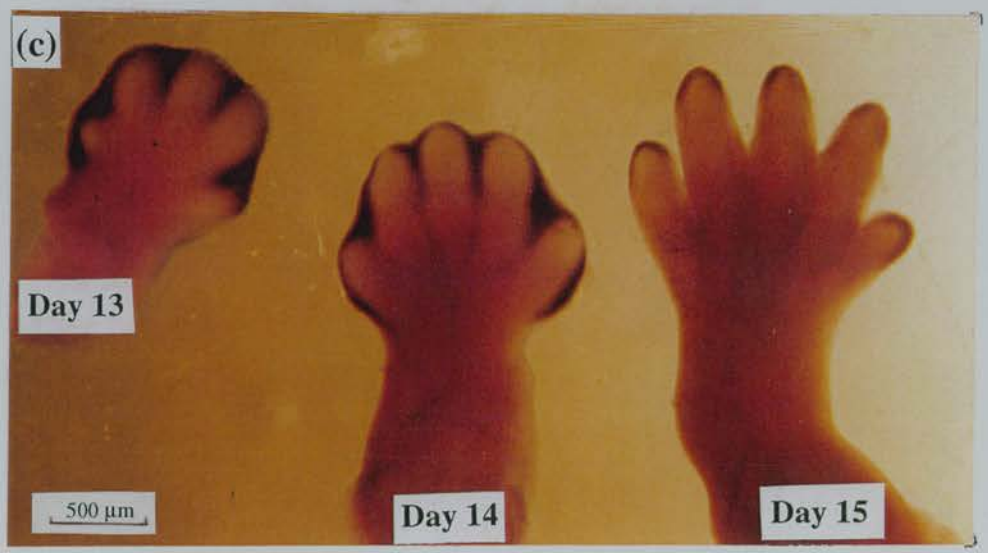
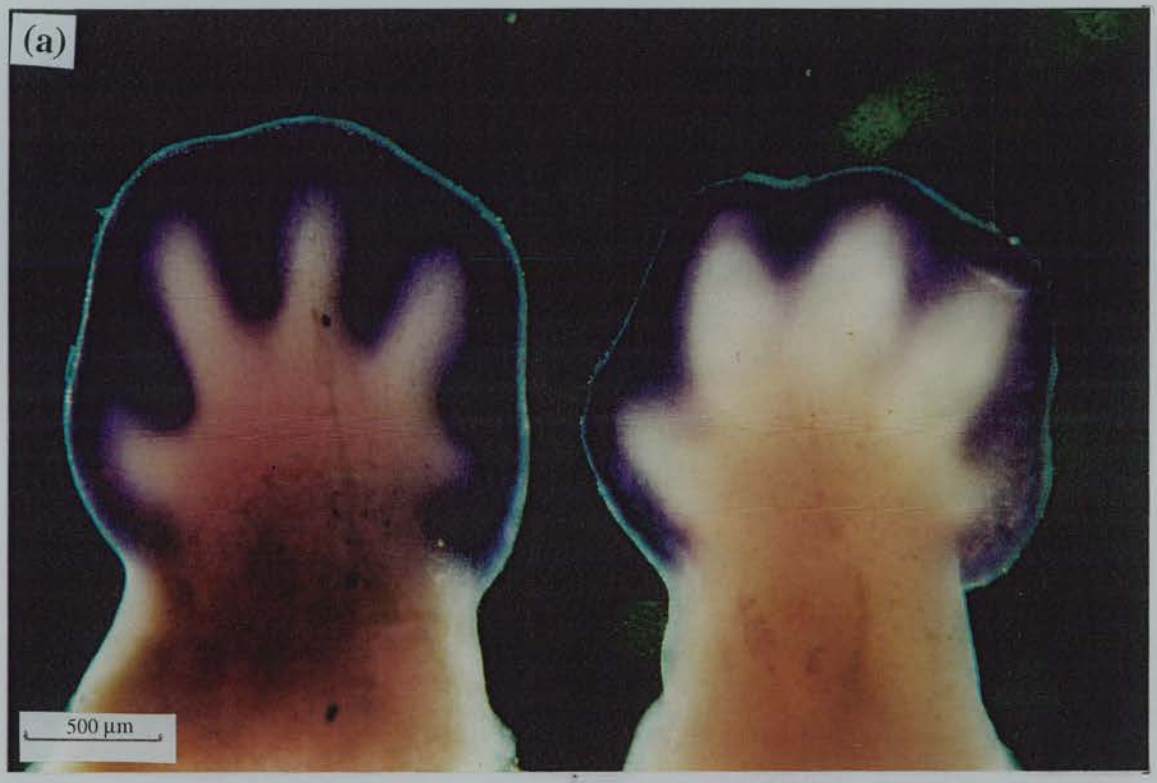

(c)
Day 13
500 µm
Day 14
Day 15

Figure 6.7 Representative photographs showing the expression pattern of *msx-1* gene using whole mount in situ hybridization in the hindlimbs isolated following the ASP procedure.

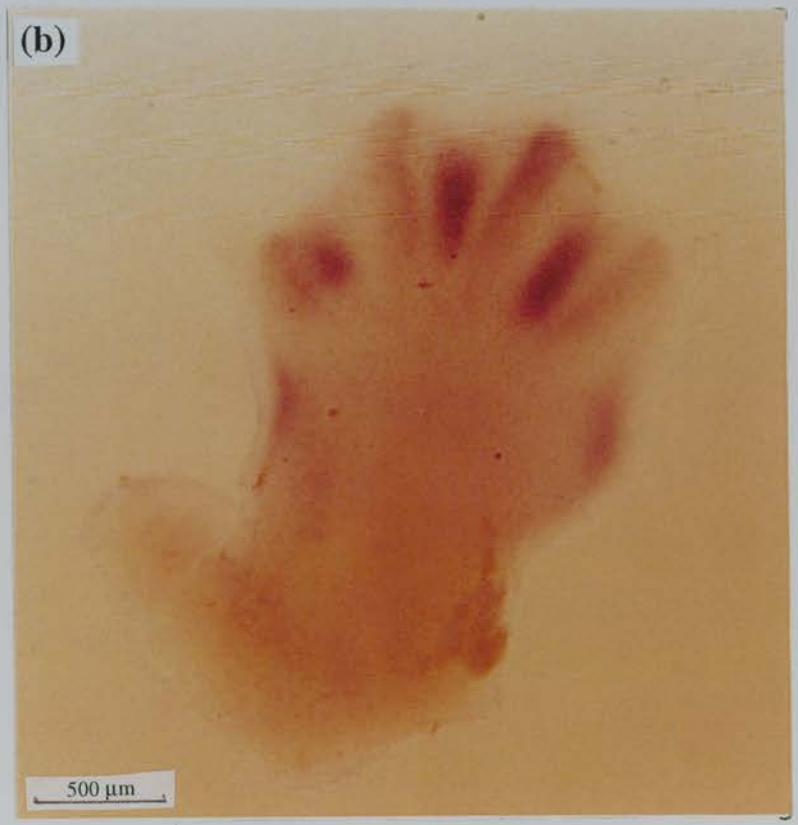
(a) comparison of the expression pattern of *msx-1* between the "internal" control and experimental hindlimb within the same litter isolated 4 hours following ASP. Right: the "internal" control hindlimb. A comparable expression pattern to that shown in Figure 6.6a is observed. Left: the experimental hindlimb. Highly intensive expression pattern of *msx-1* gene is encountered in this hindlimb. The expression extends towards to the proximal parts of the interdigital zones. Also, the expression extends to the digital areas.

(b) the expression pattern of *msx-1* in a hindlimb isolated form a dead embryo in the ASP + 12h group. The expression pattern is only restricted to the middle areas of the interdigital zones.



ASP + 4h (experimental)

Without ASP + 4h ("internal" control)



ASP + 12h (experimental; dead)

Chapter 7: Influence of anaesthetic agent on limb abnormalities observed following anmiotic sac puncture

7.1 A brief review of previous data

As described in the previous chapters, a high incidence of palatal, limb and tail abnormalities was consistently produced in this mouse model following ASP carried out on the morning of day 13 of pregnancy when Avertin was used as the general anaesthetic. In regard to the aetioloty of palate defects observed, it has been sugested that these have a postural basis due to the compression of the extraembryonic membranes and the uterine muscles (full evidence see section 1.3.3.1). Distal limb defects including adactyly, syndactyly and brachysyndactyly were commonly encountered. The aetiology of the latter, however, was believed to have a more complicated aetiology than due to posture alone. Therefore, further studies were undertaken to investigate the underlying mechanism of these characteristic defects observed in our model. In a subsequent analysis to investigate the skeletal details, the findings showed that in most cases the cartilaginous and bony elements were all present. No evidence of fusion between digital elements was found even in the cases of adactyly, suggesting that the latter abnormalities involved exclusively the soft tissues, rather than the skeletal elements.

It had been suspected that the high incidence of soft tissue syndactyly and other similar abnormalities had a vascular basis, though the exact underlying mechanism was unclear. This hypothesis was based on the fact that areas of petechiae or cutaneous haemorrhage were usually observed on various parts of embryos following ASP in our own studies and those of others. Analysis of the heart rate was undertaken and the findings confirmed this hypothesis. A general effect of bradycardia was observed in the embryos after ASP. To our surprise, embryos isolated from the females who received the anaesthetic agent Avertin (details see section 2.2.2.1) *alone* also displayed bradycardia for an average period of two hours. In our amended hypothesis, we believe that the additional duration of bradycardia detected in the ASP experimental groups may induce the limb abnormalities observed in our studies. In the latter groups, a total period of 4 hours of bradycardia was identified, lasting for an extra 2 hours in comparison with the "anaesthetic-only" control groups This prolonged duration, together with the compression by the extraembryonic membranes and uterine muscles, may generate vascular (venous) stasis and result in the soft tissue syndactyly and other similar abnormalities seen in this model.

7.1.2 Introducing a new anaesthetic agent

Based on our previous findings, it has been suspected that if the *increased* duration of bradycardia in the experimental embryos could be reduced to a minimal level, then the incidence and type of defects

observed might be altered. To fulfil the latter proposal, a different anaesthetic agent was introduced. This was based on the assumption that a prolonged post-operative recovery duration in the mothers would inevitably augment the period of bradycardia observed in these embryos. In our previous studies, an intraperitoneal injection of Avertin (details see section 2.2.2.1) was consistently administered to the females as the anaesthetic agent. Following operation, it usually took the females between 30-90 minutes to become fully recoverd under the optimal post-operative environment. After consulting the chief animal technician in the medical faculty area (Mr. D Henderson), an inhalational approach was recommended. Under the administration of a gaseous mixture of halothane, oxygen and nitrous oxide in a ratio of 2:3:3, the females were operated on as previously and returned to a similar environment used previously for recovery. According to the information supplied by the manufacturer, following administration of this new anaesthetic, it should only require 3-5 minutes the most for the females to achieve a complete recovery after operation. It was expected that with this considerable reduction of post-operative recovery time, the influence of vascular (venous) stasis would be considerably decreased, and consequently alter either the incidence or the type of the abnormalities encountered previously in this model. It was also planned that since the only modification of this experiment was the employment of a different anaesthetic agent, data obtained from the present study would be comparable to those presented in the previous one (MacIntyre et al., 1995).

7.2 Materials and Method

7.2.1 Mouse (details see section 2.2.1)

7.2.2 Anaesthetic (Avertin) (details see section 2.2.2.1)

7.2.3 Amniotic sac puncture (details see section 2.2.2.3)

7.2.4 Analysis of experimental findings (details see sections 2.2.2.4 and 2.2.2.6)

7.2.5 Anaesthetic (halothane: inhalation approach)

A gaseous mixture of halothane (manufacturer: May & Baker), oxygen and nitrous oxide in a ratio of 2:3:3 was generated by a fluotec 3 system machine purchased from INM (International Marketing Ltd). This machine consists of a simple valve and cylinder system, an attached anaesthetic chamber and associated inhalation tubing. The main part of the machine was obtained from the above company, while the chamber and tubing was constructed by the animal technicians. The valve and cylinder system is designed to provide each gas component accurately and to compose an anaesthetic agent with the proper rates of each component. The constitution of the anaesthetic mixture was then introduced to the attached anaesthetic chamber in which the females were initially placed. The animal was under a close observation through the transparent cover of the chamber. As soon as the animal became immobilized, it was

immediately removed to the operating table where the inhalation tubing could be applied immediately. Meanwhile, the strength of the gaseous mixture was reduced to an anaesthetic level. The operation was then performed with a continuous administration of the inhalation anaesthetic. Once the operation was completed, the animal was transferred to a special container for recovery. The container was equipped with two facilities for the recovery purpose. These includes a delicately designed ventilating plate inserted on the top of the lid, and a electronic heating pad fitted on the bottom of the container. The heating pad was always pre-heated to 35-37° C, and remained within this range by applying an automatic temperature controller. After placing the anaesthetised animal inside this container, a digital thermometer was placed immediately next to the animal to provide an additional safeguard system. It usually took no more than 3-5 minutes for each animal to complete the post-operative recovery. Following their recovery, the animals were retained in this container for a further 30 minutes and finally returned to the cages, where they were kept until the required time for investigation.

7.2.6 Statistics

To analyse the data produced from the present study statistically, student's t tests were applied. The chi square test and two-sample t-test were conducted whenever possible to compare the information obtained between the present and previous study (MacIntyre *et al.*, 1995; also see chapter 2) in which ASP was carried out under intraperitoneal Avertin anaesthesia.

7.3 Results

According to the experimental design, the only difference between the present and previous study was the administration of various anaesthetic agents. This, therefore, allows us to compare the observations directly between these two studies.

7.3.1 Survival rate as determined on day 19 of pregnancy when amniotic sac puncture was carried out during the morning on day 13 of pregnancy

In the current experiment, a total of 13 females were studied. Under halothane inhalation, ASP was performed on a total of 64 embryos. Within the latter, 42 embryos survived to day 19 of pregnancy when they were isolated, giving a survival rate of 65.6%. While in the previous study, where intraperitoneal Avertin was administered as the anaesthetic agent, a total of 21 females were operated. From them, 102 embryos received ASP, and 54 of them survived day 19 of pregnancy when isolated. A lower survival rate (54%) was therefore observed in the previous study, and the difference in the survival rate between these two experiments was significant.

On day 19 of pregnancy, from the 'internal' control uterine horns, 46 and 86 embryos were recovered respectively from the current and earlier investigations. A survival rate of 98% and 97% was achieved in these 2 studies. Careful analysis revealed that no morphological anomalies involving the palate, limb and tail was observed in the 'internal' control embryos in either of these studies.

7.3.2 Incidence of cleft palate

Within the 42 live embryos isolated from the experimental groups on day 19 of pregnancy in the current study, 15 displayed no morphological anomalies. Of the other 27 embryos, one or more mophological defects involved various systems was noted. Out of the latter embryos, 16 (38.1%) exhibited a cleft palate. The incidence of cleft palate was slightly higher in the current study when compared to the previous one. In the experimental group of the earlier study, 19 out of 54 (35.2%) embryos analysed on day 19 of pregnancy displayed a cleft palate. The difference in the incidence of cleft palate between the two studies is significant.

7.3.3 Incidence of all limb abnormalities observed

Details of gross abnormalities of all the forelimbs and hindlimbs from these 42 surviving embryos were recorded. Out of 168 (42 x 4) forelimbs and hindlimbs examined, 79 (47,0%) exhibited at least one type of limb abnormality (for illustration, see Figure 2.3). The overall incidence of limb defects in the current study was significantly higher than that of the previous one. When 216 forelimbs and hindlimbs in the previous study were analysed, 67 (31.0%) displayed abnormalities of one form or another.

A list of detailed digital abnormalities encountered in the current study was provided in Table 7.1, along with that of the previous report.

7.3.3.1 Incidence of syndactyly

To our surprise, unlike the overall findings, the incidence of syndactyly displayed an opposite result. Syndactyly was observed in 26.6% (21 out of 79) of limbs in the current study. This frequency was considerably lower when compared to the previous study, in which almost two-thirds of the abnormal limbs studied (47 out of 67, 70.2%) exhibited syndactyly.

7.3..3.2. Incidence of postural limb abnormalities

In contrast to the syndactyly findings, the incidence of postural limb abnormalities increased dramatically in the present compared to that in the previous study. A range of deformities, though similar in type in both studies, was observed in the affected limbs. In the forelimbs, club hand and drop wrist (definition see section chapter 2) were commonly seen, while in the hindlimbs, club foot (talipes) were usually encountered. Out of 79 affected limbs, 38 (48.1%) displayed a postural deformity in the current study. The incidence was much lower in the previous study; in 67 affected limbs, only 13 of them (19.4%) had a postural limb deformity.

7.3.4 Incidence of tail abnormalities

Although a similar range of abnormalities was observed, the overall incidence of tail abnormalities seen in the present study was dramatically reduced. Only 3 out of 27 tails (11.1%) showed morphological defects. This contrasted with the previous findings, where out of 38 embryos with morphological abnormalities, a total of 23 (60.5%) were found to have tail abnormalities. It is worth noting that all those 3 embryos that displayed tail abnormalities in the current study had cleft palates, and about half (52.2%) of the embryos with tail abnormalities in the previous study also presented with a cleft palate.

7.3.5 Mean weight of (i) morphologically abnormal, and (ii) morphologically normal 'experimental' embryos.

After examining their gross morphology, the experimental embryos were immediately subdivided into two groups: (i) those that presented any abnormalities of either palate, limb or tail. (ii) those that showed no obvious gross abnormalities. A detailed study of these two groups in the current and previous study is presented in Table 7.2. The body weight of each embryo in these groups was tabulated and a mean value was calculated within each group. The results revealed that in both the current and earlier studies, those embryos with one or more abnormalities in group (i) inevitably weighed less than the normal embyos in group (ii). However, while the statistical difference in mean body weight between groups (i) and (ii) was significant in the present study, it was not statistically significant in the previous study. Table 7.2 and 7.3 presenting information relating to body weight and their relationship between groups (i) and (ii) in both studies.

7.3.6 Mean crown-rump length of (i) morphologically abnormal, and (ii) morphologically normal 'experimental' embryos.

When the crown-rump length of each embryo was recorded and the statistical analysis applied on groups (i) and (ii) according to the definition described above (section 7.3.5), the results showed a similar relationship between the grossly normal and abnormal groups. In those embryos with any form of abnormalities, a reduced length was observed compared to those which displayed a normal morphology. Again, the statistical relationship was similar in both current and previous studies, although a greater difference between groups (i) and (ii) was encountered in the current study.

7.4 Discussion

It was our aim to test whether the anaesthetic agent employed might influence the incidence and type of abnormalities observed. In our previous studies (MacIntyre *et al.*, 1995; Chang *et al.*, 1996; Chang and Kaufman, 1997), we exclusively used an intraperitoneal injection of Avertin. When this agent was administered it usually took 30-90 minutes for the animals to fully recover after the ASP operation. In this study, an inhalation of halothane mixture was applied, and the females recovered from the anaesthetic a much faster post-operative recovery (i.e. within 3-5 minutes) compared to when Avertin was used. While other operative procedures remained essentially identical, a comparion between the current and previous studies allowed us to investigate the influence of these anaesthetic agents on the incidence and type of limb abnormalities encountered. The results clearly demonstrate that various anaesthetic agents may produce different outcome on the incidence and type of limb abnormalities observed.

As might have been expected, after a substantial reduction in the duration of the post-operative recovery time, the outcome of the ASP was also changed considerably between the current and previous studies. Firstly, the survival rate in the ASP-exposed embryos highly significantly increased in the present study. Secondly, the incidence of cleft palate significantly increased, though the difference was considerably less marked. Thirdly, the incidence of the overall limb abnormalities also significantly higher. In the classification of the limb abnormalities, the incidence of syndactyly, however was significantly reduced, while the incidence of postural deformities was surprisingly substantially higher. Fourthly, the incidence of tail abnormalities was significantly decreased, only being seen in 3 out of 27 affected embryos. Fifthly, although similar findings of mean body weight and mean crown-rump length were observed between the morphologically grossly normal and abnormal embryos in both studies, a significant difference was only found between these groups in the current study.

We have previously suggested that the aetiology of the limb abnormalities observed following ASP may have two principal explanations, having either a postural basis, or a vascular basis, or the combination of the two. We anticipated that by altering the anaesthetic agent and reducing the recovery duration this would diminish the bradycardia-induced vascular disruption observed following ASP. The general improvement in the survival rate of the ASP-exposed embryos in the current study confirmed this possibility. In addition, the reduced incidence of syndactyly and tail abnormalities in the present study might have been a consequence of the much briefer duration of vascular disruption. Although the underlying mechanism has yet to be established, the most plausible explanation of the pathogenesis of the syndactyly and other limb abnormalities observed strongly suggested that it might be due to vascular (venosis) stasis as a consequence of compression by the extraembryonic membranes and the uterine muscles. This hypothesis was mainly based on the high incidence of haemorrhagic lesions observed on the extremities of the embryos shortly after ASP undertaken under Avertin anaesthesia. The influence would be greater when a prolonged period of embryonic bradycardia was generated by the anaethetic agent administrated to the mother in order to perform ASP. Although no *direct* evidence could be demonstrated in the current study, namely that the inhalation of halothane would shorten the period of embryonic bradycadia (as time was not available to perform this experimental study), it is believed that this is the most likely explanation. Based on the latter assumption, the vascular disruption provoked by the intraperitoneal injection of Avertin was almost completely eliminated, leaving the influence of compression alone on the embryo as the major teratogenic influence.

In the previous study, carried out under the Avertin anaesthethia, it was strongly suspected that prolonged duration of bradycardia could result in temporary hypoperfusion in the interdigital zones. This probably subsequently interrupted the normal pattern of programmed cell death (i.e. apoptosis) that normally occurs in this region during digit formation, and consequently increased the incidence of syndactyly. While in this study, under the inhalational anaesthetic agent was used, it was believed that only a much briefer duration of embryonic bradycardia would be induced. Thus, the interference of the metabolic procedure and/or the cellular environment in the interdigital zone would be limited to a relatively restricted period, allowing the normal progress of apoptosis in this area, and consequently decreasing the incidence of syndactyly in the current study.

It was suggested that a similar explanation might account for the reduced incidence of tail abnormalities observed in the current study. During tail elongation, the tail is relatively well protected by the caudal part of the body, thus when the amnion collapsed following ASP, there should be still reasonable space to allow the tail development and a minimal incidence of tail abnormalities was observed. Therefore the high incidence of tail abnormalities observed in the earlier study and the haemorrhagic lesions involving the distal part of the tail observed in other reports (Houben, 1984) led us to believe that the tail

abnormalities observed following Avertin ASP was also likely to be associated with a vascular aetiology. The extremely low incidence of tail abnormalities in the current study confirmed the latter argument, though indirectly.

While the incidence of the abnormalities believed to have a vascular aetiology dramatically declined in this current halothane ASP study, the incidence of those with a postural basis surprisingly appeared increased. The difference between the current and earlier study was modest in regard to the incidence of cleft palate, but remained marked in the situation of overall limb abnormalities and its subdivision, particularly with regard to limbs with postural defects. These findings indicated that although the teratogenic effects related to a prolonged recovery time required after the injection of Avertin has been largely prevented, the adverse effects of ASP alone might still be present regardless of whichever anaesthetic agent was employed. One possibility exists that, without an adequate quantitive study after proper staining to reveal the skeletal element, errors may occur in these two separate studies. Further investigation using cleared and stained skeletons would help to clarify this aspect.

We are convinced that the findings reported in this study offer confirmatory, though indirect, evidence of a likely mechanism of induction of syndactyly following Avertin ASP. Additionally, an identical study to determine the embryonic heart rate following ASP, should be undertaken by using inhalation halothane as the anaesthetic agent instead of intraperitoneal Avertin. This further investigation would allow us to demonstrate that the inhalational anaesthetic agent alone only was responsible for producing a limited period of embryonic bradycardia. Consequently, a much reduced rather than a delayed and prolonged period of bradycardia might reasonably be expected to be observed in the embryos following halothane ASP.

The major interest in this study was to clarify the mechnism of induction of the abnormalities with either a postural or a vascular aetiology following ASP by using various anaesthetic agents. We also believe that the findings in the current study provide an indication for future experiments. Studies performed on pregnant animals using general anaesthetic may have a *secondary* effect, namely embryonic bradycardia, and that this would inevitably influence the experimental outcomes, particularly if operative intervention occurred at the time prior to and/or during organogenesis.

Table 7.1 Various limb abnormalities noted in experimental embryos analysed on day 19 of pregnancy . Amniotic sac puncture (ASP) was performed on day 13 of pregnancy in the present study, using inhalational halothane, and in the previous study, using Avertin (results published in MacIntyre *et al.* 1995).

Study	Limb	Limb affected (%)	Adactyly	Syndactyly	Brachysyndactyly	Diastodactyly	Missing first digit	Postural abnormalities*
1. present study (halothane)	LFL	26 (33)	3	5	3	5	2	8
	RFL	20 (25)	0	1	1	4	1	13
	LHL	19 (24)	1	6	3	0	0	9
	RHL	14 (18)	1	2	0	3	0	8
	Total	79 (47)	5	14	7	12	3	38 (48 %)
2. MacIntyre *et al.* (1995) (Avertin)	LFL	11 (16)	2	4	2	0	0	3
	RFL	12 (18)	0	4	3	0	0	5
	LHL	25 (37)	1	10	7	3	0	4
	RHL	19 (28)	0	7	10	1	0	1
	Total	67 (31)	3	25	22	4	0	13 (19 %)

LFL, left forelimb; RFL, right forelimb; LHL, left hindlimb; RHL, right hindlimb
* postural abnormalities includes ulnar/radial deviation and/or "clubhand" in the forelimbs; talipes equinovarus or "clubfoot" in the hindlimbs

Table 7.2 Comparison of crown-rump length between two experimental groups and one control group. Experimental embryos are divided into two groups, those with no obvious external abnormalities (Normal expt.), and those with one or more external abnormalities (Abnormal expt.). Control embryos are those from "internal" control group (definition see section 3.2.1). Inhalational halothane was employed in the present study and an intraperitoneal injection of Avertin was used in the privious study reported in MacIntyre *et al.* (1995).

Crown-rump length (Mean +/- S.E.M. mm)

	Normal expt. #.	Abnormal expt.*	"internal" controls #
1. present study (halothane)	22.07 +/- 0.318 (n = 15)	19.55 +/- 0.432 (n = 27)	22.96 +/- 0.216 (n = 46)
2. MacIntyre *et al.* (1995) (Avertin)	21.53 +/- 0.374 (n = 16)	20.41 +/- 0.288 (n = 38)	22.67 +/- 0.165 (n = 83)

* when the data between the present and previous studies were compared, the differences were significant, $p<0.05$; in the other comparisons (designated as #), the differences were not significant, $p>0.05$

Table 7.3 Comparison of body weight between two experimental groups and one control group. Experimental embryos are divided into two groups, those with no obvious external abnormalities (Normal expt.), and those with one or more external abnormalities (Abnormal expt.). Control embryos are those from "internal" control group (definition see section 3.2.1). Inhalational halothane was employed in the present study and intraperitoneal injection of Avertin was used in the privious study reported in MacIntyre *et al.* (1995).

Weight (Mean +/- S.E.M. g)

	Normal expt. #.	Abnormal expt.*	"internal" controls #
1. present study (halothane)	1.160 +/- 0.020 (n = 15)	1.020 +/- 0.028 (n = 27)	1.214 +/- 0.012 (n = 46)
2. MacIntyre *et al.* (1995) (Avertin)	1.072 +/- 0.019 (n = 16)	1.069 +/- 0.021 (n = 38)	1.133 +/- 0.011 (n = 83)

* when the data between the present and previous studies were compared, the differences were significant, $p<0.05$; in the other comparisons (designated as #), the differences were not significant, $p>0.05$

Table 7.4 Comparison of crown-rump length and body weight between two experimental groups and one control group‡‡ in the present study **.

	Abnormal expt.	Normal expt.	Control
Abnormal expt.	---------	$p<0.01$	$p<0.01$
Normal expt.	$p<0.01$	--------	$\underline{p>0.05}$
Control	$p<0.01$	$\underline{p>0.05}$	--------

The underlined p values indicate that no significant difference was found at the 5% level.
‡‡ As defined in table 7.1 and 7.2
** The same result was found for both the crown-rump length and body weight when compared between/within each group.

Chapter 8: General discussion

This chapter aims to (1) explain the reason why the mouse ASP model was established, (2) review the findings in this study, (3) discuss the mouse ASP model in comparison with the human CVS clinical findings and the suitability of this model in studying limb development, (4) discuss in general terms the possible underlying mechanisms that cause the abnormalities found in the present study, and (5) suggest future experiments that can be of immediate relevance to this study.

8.1 The reason for establishing pioneer animal models for ASP -- concerns about the safety of amniocentesis

About four dacades ago, the first clinical procedure to determine the human fetal sex using desquamated fetal cells in the amniotic fluid was developed and termed amniocentesis (Dewhurst, 1956; Makowski *et al.,* 1956; Sachs *et al.,* 1956). The procedure proved to be a successful prenatal diagnostic approach. Certain risks associated with amniocentesis, however, were noted in the first report and the author (Dewhurst, 1956) stated that "*the procedure was unsuitable for general use to discover a child's sex which would soon be known for certain*".

8.1 Earlier animal studies for amniotic sac puncture and their findings

Trasler and colleagues (1956) first conducted a series of animal studies to investigate the traumatic effects of amnion rupture on developing mouse embryos. Demonstrating a high incidence of cleft palate in the study, they claimed "*Our results with mice suggest that there may be a definite risk to the baby in inserting a needle into the amniotic sac in human beings, especially during the early stages of pregnancy when there is a danger of inducing abnormalities in the developing embryo.*"

In the earlier animal ASP studies, both in the mouse and rat, the finding of cleft palate was the greatest cause of concern, and its etiology was extensively studied both macroscopically and microscopically (mouse: Trasler *et al.,* 1956; Walker, 1959; rat: Poswillo, 1966). A clear explanation of the aetiology of ASP-induced cleft palate was provided, and said to be due to the compression of the embryo by uterine muscles and extraembryonic membranes, as a result of reduction of hydrostatic pressure in the amniotic sac following ASP. It was suggested that in the latter circumstances, the embryo was constricted, forcing the head and the lower jaw against the chest, retaining the tongue between the palatine shelves (i.e. as the mandible was prevented from descending, this prevented the palatal shelves from elevating from the vertical to the horizontal position as they were at this stage of development located on either side of the tongue), and resulting in cleft palate. Supplementary evidence to support the latter hypothesis was also observed in a series of studies of movement of embryos (Walker, 1959, 1967, 1968). It was suggested that the extension of the neck region was

fundamental at the time of palate closure. This movement would lift the head away from the chest and yield plenty of room for the tongue and the lower jaw to open and close. Their results demonstrated that embryos with reduced neck reflex movements were usually associated with cleft palate and cleft lip (Walker, 1969), suggesting that the postural compression of the neck region onto the chest due to the force of the uterine muscles and extraembryonic membranes following ASP also contributed an important role in cleft palate formation.

Poswillo (1966) observed that the type of cleft palate induced by the ASP was morphologically similar to the human palate defect, when compared with those induced by other teratogens in previous animal studies. Other characteristic features such as micrognathia (abnormally small size of the lower jaw) and glossoptosis (downward retraction of the tongue) were also noted at the same time when cleft palate was observed in these rat embryos. Therefore, Poswillo (1966) suggested that the ASP syndrome was analogous to the human Pierre Robin syndrome (Robin, 1923, 1929; Jones, 1988), in which the triple features of cleft palate, micrognathia and glossoptosis were usually observed simultaneously. Although the contemporary classification of the human Pierre Robin syndrome does not include limb defects any more, this initial observation by Poswillo (1966) indicated a strong relationship between craniofacial and limb abnormalities. Additional observations were consistently noted in the subsequent rat ASP studies (DeMyer and Baird, 1969; Love and Vickers, 1972; Singh and Singh, 1973; Singh *et al.,* 1974; Kino, 1975; Kennedy and Persaud, 1977; Houben, 1980, 1984; Houben and Huygens, 1987). In the mouse, such a relationship was not reported in the earlier ASP studies (Trasler *et al.,* 1956; Walker, 1959). However, it was well documented during the establishment of our mouse model (MacIntyre *et al.,* 1995).

While these earlier ASP studies were largely interested in the craniofacial defects induced by ASP, and many authors provided a postural basis to explain these phenomena, in the more recent studies in the animal ASP series, limb defects were also noted and their aetiology proposed accordingly (DeMyer & Baird, 1969; Love & Vickers, 1972; Singh & Singh, 1973; Singh et al., 1974; Kino, 1975; Kennedy & Persaud, 1977; Houben, 1980, 1984; Houben & Huygens, 1987). Immediate and prolonged signs of a disrupted vascular system were observed in the extremities, as well as on the head and trunk region after ASP in the rat. As short as 15 minutes following the ASP procedure, extensive areas of cutaneous bruising were usually found in the treated embryos. This was particularly evident on the distal part of the limbs. Detailed examination on the limbs at daily intervals after ASP revealed that haemorrhages, vascular dilatation and congestion as well as damaged endothelium of blood vessels were frequently observed in the marginal vein and in the capillaries in the interdigital zones, where most of the limb defects were subsequently encountered. It was therefore suggested that vascular disruption in various forms following ASP in these studies had been a major underlying cause of the induction of the limb defects seen in these treated embryos.

8.1.2 Comparable gestational stage in human embryos when ASP is performed in animals

It is worth noting that, although the latter series of animal ASP studies were originally designed to examine the teratogenic effects of amniocentesis on the developing embryos, the ASP procedure was actually performed slightly earlier in these animal studies when compared to the time amniocentesis would normally be carried out clinically. For example, in the earlier mouse ASP studies (Trasler *et al.*, 1956), the puncture was introduced at a developmental stage equvalent to about 50-51 to 56-57 days post-ovulation, or Carnegie Stages 20-23 of *human* embryonic development (Kaufman, 1992; O'Rahilly & Müller, 1987). Clinically, amniocentesis is normally performed between 12 to 16 weeks' gestation, thus it was obvious that these animal ASP studies had been carried out at a rather earlier stage, although the technique applied in both human and in the various animal studies were similar. Therefore, it is our view that these ASP animal studies were more relevant to one other prenatal diagnostic procedure, namely *chorionic villus sampling* (CVS), a diagnostic technique which was not developed for routine use until relatively recently (details see 1.2.1).

8.2 Clinical findings following CVS and their relevance to the animal studies

It is surprising that the possible relevance of these earlier animal ASP studies were largely ignored and no precaution was taken by the operators during the early of CVS clinical trials. Consequently, various complications and unexpected fetal abnormalities were encountered in otherwise genetically normal fetuses in many clinical series. Had they been fully aware of the experimental findings, it is likely that many of the unanticipated fetal abnormalities might have been prevented. However, the lack of awareness of the existence of these relevant animal studies may be due to the fact that the latter studies were performed to investigate the teratogenic effects of *amniocentesis*, although the ASP procedure was undertaken at an equivalent time during pregnancy when CVS would normally be carried out clinically.

8.2.1 Safety and associated complications encountered following CVS

Pregnancy loss rates following CVS was reported to be between 2% and 3% before 28 weeks of gestation (Jackson *et al.*, 1992), although lower rates had previouly been reported by other centres (Clark *et al.*, 1989; Green *et al.*, 1988; Gustavii *et al.*, 1989; Jahoda *et al.*, 1989; Young *et al.*, 1991).

Vaginal bleeding, uterine infection, and rupture of the membranes were common complications following CVS. As many as 7% of women suffered vaginal bleeding following transcervical CVS; 2% after transabdominal CVS (MRC Working Party, 1991; Rhoads *et al.*, 1989).

Evidence of acute rupture of the membranes resulting in amniotic fluid leakage and/or the presence of a reduced quantity of amniotic fluid later during the pregnancy was subsequently studied by ultrasonography (MRC Working Party, 1991; Rhoads *et al.*, 1989). Delayed rupture of the membranes, due to either mechanical insults or bacterial infection involving the chorion, was reported to be between 0.3% (Brambati *et al.*, 1987; Hogge *et al.*, 1986; MRC Working Party, 1991), and 0.7% (Rhoads *et al.*, 1989). Amazingly, *unexplained* midtrimester oligohydramnios has also been occasionally reported as one of the complications of CVS (Cheng *et al.*, 1991).

8.2.2 Abnormalities induced by CVS

After reviewing all these complications of CVS, doubts remained as to whether aspects of the technique of CVS might have any detrimental effects on the development of the embryo. However, since the major concern with regard to safety was largely concentrated on two aspects, namely *maternal welfare* and *pregnancy losses*, very little attention was given to the potential teratogenic role of CVS on these embryos. Therefore, it was a great surprise for both the clinicians and the mothers when infants were born with unexplained craniofacial and limb defects following CVS (reports in the literature, see section 1.3.2). Firth and her colleagues (Firth *et al.*, 1991a) were the first to raise the possible association between craniofacial and limb abnormalities and CVS when this procedure was undertaken during days 56-66 of gestation. Out of 289 babies who were exposed to CVS during the stage indicated above, 5 of them had craniofacial and limb abnormalities comparable to those observed in the OMLHS syndrome (for details see section 1.3.1), namely cleft palate, cleft lip, micrognathia, glossoptosis, associated with an array of limb defects ranging from distal amputations, transverse limb reduction defects to hypoplasia of the finger nails.

Following the report by Firth *et al.* (1991a), clusters of clinical cases appeared in the literature and large scale surveys were conducted to test whether there might be an association between CVS and the induction of craniofacial and limb defects. Different studies, however, showed results that were not consistent with each other. The inconsistency observed between these studies was probably largely due to the different criteria used to evaluate the findings (see section 1.2.6.2 for details). The debate as to the possible mechanism of CVS-induced abnormalities in the craniofacial and limb regions had continued until relatively recently when properly designed surveys were conducted and the criteria re-evaluated (Hsieh *et al.*, 1995; Botto *et al.*, 1996). Most authorities now accept that CVS may cause limb and craniofacial abnormalities (Wapner, 1997).

8.2.3 Possible explanations for the abnormalities

Various hypotheses have been proposed by many researchers to explain the aetiology of the OMLHS syndrome following CVS when this procedure is carried out during early gestation (details see 1.3.3 -

1.3.3.4.2). In the rat studies, similar defects were produced simply by clamping the uterine vessels for 45 minutes (Brent & Franklin, 1960) or for up to 3 hours (Brent & Franklin, 1960). Moreover, similar defects were also noted in the studies in which normal uterine blood flow was deliberately reduced by applying various degrees of vasoconstriction (Leist & Grauwiler, 1974). Accordingly, it has been suggested that a temporary reduction of the fetal blood circulation might induce hypoxia, hypoperfusion, bradycardia and serum ion changes in the fetuses, resulting in hypoplasia, amputation, necrosis and resorption of parts.

Clinically, it appeared to be more difficult to explain the abnormal findings following CVS, considering the fact that the procedure of CVS itself only involved collecting a small sample from the developing placenta, without contacting the embryo *per se*. Several divergent mechanisms to explain the relationship between OMLHS syndrome and CVS have been proposed, although the majority of the authors were in favour of a vascular aetiology.

Before CVS became largely available in clinical practice, microscopic examination of the placenta of four fetuses born with unilateral transverse limb reduction defects revealed that multiple centres of organized thrombi were observed over most of the large mainstem fetal villous vessels (Hoyme et al., 1982). The authors strongly suspected that thrombus formation within the placental bed could have played an important role in the production of these defects. To test whether placental damage *per se* could induce haemorrhagic lesions of the fetus, partial placental detachment and subchorionic haematomas were deliberately produced using a blunt instrument during CVS in seven cases shortly before their therapeutic termination of pregnancy (Quintero *et al.*, 1992). Although general haemorrhagic lesions all over the fetuses were observed by embryoscopy, no signs were observed of similar vascular damage were located on the fetal limbs.

Several other causes of vascular disruption during CVS were proposed (details see section 1.3.3.2 and 1.3.3.3). Firstly, it was proposed that the damage at the biopsy site may result in thrombus formation within the local vessels and generate hypotension, embolisation, hypoperfusion, or anoxia in the distal extremities, or alternatively, the placnetal injury may stimulate the secretion of vasoactive factors into the fetal circulation (Firth *et al.*, 1991b). Secondly, it was suggested that the exocoelomic gel (which is located between the chorion and amnion, and which is obliterated by about day 56 - 63 of gestation) might be relevant to the digital lesions observed in these infants due to the possible entrapment of one or more extremities into the obliterated exocoelomic gel; this might result in hypoxia and/or tissue necrosis in these areas (Shepard *et al.*, 1991). This hypothesis was, however, unable to explain the abnormalities observed in the craniofacial region. Thirdly, when maternal cells were found in 20% of the CVS-exposed infants, the possibility was raised that maternal antigens may be introduced into the fetal circulation during CVS (Los *et al.*, 1996). To test the latter hypothesis, maternal antisera were injected into the 10-somite stage rat embryos maintained in tissue culture (van der Zee *et al.*, 1997).

Based on the fact that the dorsal aorta possesses an intact endothelial lining while the pharyngeal arch arteries and the limb vessels still possess a fenestrated epithelium at this stage of development, the authors suggested that the maternal antisera may disrupt the blood supply to these locations with resultant craniofacial and limb defects. Doubts, however, remains since the immunological status of these embryos is far less than those exposed clinically to CVS, and that this model may not properly represent the situation observed in the fetuses who normally receive CVS.

From our point of view, none of these hypotheses was entirely satisfactory and able to explain both the craniofacial and limb abnormalities observed following CVS. We believe that the most plausible explanation is that inappropriate rupture of the amnion membrane may occasionally be induced during CVS (Shepard *et al.*, 1991), resulting in oligohydramnios and fetal compression from the uterine muscles and the extraembryonic membranes, subsequently inducing hypoperfusion and hypoxia of tissues (Kaufman, 1994). Clearly should this occur, it might cause immobilisation and postural remodelling of the embryos. Thus, *both* of the craniofacial and limb abnormalities observed following CVS could be explained. Based on the associated complications observed following CVS (details see section 1.2.6 and 8.2.1), we strongly suspected that the *unintentional* damage of the amnion during CVS may occasionally encountered, and that if this was associated with *early* CVS, this might lead to the fetal abnormalities observed clinically.

8.3 Establishment of the animal model for CVS-induced abnormalities

As indicated above, the inadvertent rupture of the amnion during CVS appeared to be the most plausible explanation for the craniofacial and limb abnormalities encountered following CVS. Once we decided to investigate the OMLHS syndrome induced by CVS and its underlying mechanism, we immediately adopted the idea that the amniotic sac puncture approach used in the previous animal experiments should be employed in our own studies.

8.3.1 Advantages: limited ethical issues and higher incidence of abnormalities

Clearly, it is extremely difficult to investigate this problem *clinically*, not only because the potential ethical problems involved but also because of the limited number of cases that were available for analysis. The *highest* incidence of the OMLHS syndrome that appeared among the clinical reports was only in the region of 1.7% (Firth *et al.*, 1991a). By contrast, animal models provide us with two major advantages: limited ethical problems accompanied with reasonable numbers of affected embryos and abundant material. While the amnion rupture in clinical cases occurred *unintentionally*, the amniotic sac puncture was *deliberately* induced experimentally into every individual conceptus, and the puncture was only considered successful when an uncontrolled leakage of amniotic fluid was observed.

8.3.2 Establishment our own mouse model

It was surprising to note that apart from a few earlier ASP animal studies, relatively few had been carried out in the mouse; the majority of the investigations had been carried out in the rat. Since we were much more familiar with the early development of the mouse (see Kaufman, 1992), it appeared better for us to choose the mouse as the study material. After reviewing the developmental stage in both the human and the mouse, it became evident that ASP should be performed during Theiler Stages 20 -22 (Theiler, 1989) in the mouse, approximately equivalent to Carnegie Stages 18 - 21 (O'Rahilly and Müller, 1987) in the human, with the expectation that a high incidence of the desired type of craniofacial and limb abnormalities might be achieved. When converting Theiler Stages 20 - 22 into mouse gestational ages, we therefore decided to undertake our preliminary studies on pregnant mice on days 12, 13, 14, 15 and 16 of gestation.

Full details of pregnancy losses, together with the incidence and type of craniofacial and limb abnormalities were noted. The results revealed that the later the ASP procedure was performed, the higher survival rate was achieved. While the range of limb abnormalities encountered remained fairly similar through this gestational period, the overall incidence appeared highest if ASP was carried out on day 13 of pregnancy. In order to obtain a high survival rate associated with a high incidence of abnormalities, the procedure was carried out on day 13 of pregnancy for all subsequent studies.

The needle size became an important factor to consistently produce the high incidence of abnormalities. When a 25 gauge needle was used initially, the incidence of the desired defects was extremely low. Thus a needle with a greater diameter (21 gauge) was employed in the subsequent series. Once these factors had been ascertained, our mouse model was employed to examine the craniofacial and limb defects induced by CVS, on the assumption that the latter defects were likely to be due to the teratogenic effects of amniotic fluid leakage due to the unintentional rupture of the amnion during CVS.

8.4 Justification of this mouse model for CVS

A recent international clinical trial involving 138,996 pregnancies was carried out under the supervision of WHO to determine the relationship between the craniofacial and limb abnormalities when CVS was performed between weeks 9 and 12 of pregnancy (Kuliev *et al.,* 1996). The results showed no significant difference in the incidence of the defects between treated fetuses and their matched controls. According to the findings, WHO has advised that CVS *should not be undertaken before weeks 9 of gestation.* Nowadays, the incidence of these craniofacial and limb abnormalities seen in the CVS-exposed infants has been dramatically reduced, due to the acknowledgement of the

potential risk if CVS was performed too early in pregnancy. The mechanism of how these unexpected abnormalities occurred, however, has yet to be fully understood. Because the ASP animal models provide the most convincing explanation for the craniofacial and limb abnormalities observed after CVS, we believe that this mouse model provides interesting insights into the mechanisms involved in the induction of the craniofacial and limb abnormalities observed, although in this thesis we concentrate on the aetiology of the limb defects observed.

8.4.1 Abnormalities encountered

Characteristic features of craniofacial and limb abnoramlities similar to those observed in the OMLHS syndrome following CVS were consistently produced using this mouse study. In the head region, cleft palate, cleft lip, micrognathia and glossoptosis were the most commonly encountered defects. While in the limbs, a full range of abnormalities were encountered, including both digital abnormalities and postural deformities. For the limb abnormalities noted in both the clinical and animal studies, it was observed that almost all of the defects involved only the distal part of the fetal extremities.

8.4.2 Reliability of this model

In the mouse model, the survival rate and incidence and type of the abnormalities appeared to be very consistent. When ASP was performed on day 13 of pregnancy, and the results analyzed on day 19, about 53% of the treated embryos survived. Within the latter group, 35% were found to have a cleft palate, and 61% displayed one or more morphologically abnormal limbs (chapter 2). Although most of the results were obtained before day 19 of pregnancy in the subsequent studies, consistent findings were observed within each individual study, suggesting a high level of reliability of this model.

8.4.3 Availability of adequate control series

One of the important advantages of the mouse ASP model is the readily available controls from the contralateral non-operated uterine horn. This is clearly not possible in clinical practice. While only the embryos from one uterine horn in each pregnant female were subjected to ASP, embryos in the opposite (contralateral) horn remained undisturbed and acted as "internal" controls. Additional embryos isolated at comparable gestational stages, from females who had received neither ASP nor anaesthetic agents, acted as "non-experimental" controls. Similarly, embryos that had been recovered at comparable gestational stages, from those mothers who had been subjected to anaesthetic agents alone functioned as "anaethetic only" controls. In this model, groups of "internal" controls, "non-experimental" controls and "anaesthetic only" controls were employed in appropriate numbers to act as appropriate controls for the findings observed in the experimental groups. Because of the accessibility of these various control series, we felt that it was justified to study this model further.

8.4.4 Mouse ASP model for studying limb development

In addition, over the last decade, the mechanisms and genes involved in the modelling or regenerating of the distal limb (details see chapter 6) have been extensively investigated. For example, following specification of limb pattern and regression of the apical ectodermal ridge, interference with the expression of several patterning genes that are normally retained in the interdigital region may occur as a result of ASP. A series of genes are believed to be particularly important: *Msx-1* and *2* (Robert *et al.*, 1989; Davidson *et al.*, 1991; Reginelli *et al.*, 1995), *Bmp-2, 4* and *7* (Jones *et al.*, 1991; Francis-West *et al.*, 1995; Lyons *et al.*, 1995) and *Fgfr-1* and *2* (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1992). This model inevitably has the potential to shed new light on means of evaluating the functions of these genes under normal physiological circumstances, as well as when exposed to teratogenic insults resulting from either mechanical compression or following vascular disruption.

8.5 Detailed studies of the mouse ASP model

After establishing the mouse ASP model and recognising its important roles in both investigating the characteristic abnormalities observed following CVS, and in serving as a model to evaluate the limb development under various experimental conditions, we decided to study the underlying mechanism of how these craniofacial and limb abnormalities observed following CVS might have been induced. Realising that the craniofacial defects observed following CVS probably had a postural basis (see section 1.3.3.1), we decided to concentrate our attention on determining the aetiology of the limb defects observed following ASP.

8.5.1 Limb and other postcranial facial abnormalities

Limb defects such as adactyly, syndactyly and brachysyndactyly were commonly observed in this mouse model, and it seemed appropriate to investigate their histological basis. ASP-treated embryos were firstly subjected to a "clearing" and bulk double staining technique using Alcian blue and alizarin red S to reveal the cartilaginous and bony elements, respectively.

8.5.1.1 Differentiation between a postural and soft tissue defects

Surprisingly, after clearing and the demonstration of the skeletal elements, it became apparent that in the majority of cases only the soft tissues (rather than the skeletal elements) had been affected. This was particularly evident in the case of *adactyly*, in which no obvious digits were distinguishable at the soft tissue level; in these cases all of the skeletal elements were obviously present although occasionally very minor skeletal deformities were observed. In general, apart from the low incidence

of skeletal deformities, it appeared that ASP had a minimal teratogenic effect on the skeletal elements. Measurements of the full bone and ossification length of the distal and proximal parts of the limb showed no significant difference, comfirming the latter hypothesis. Only in a relatively few cases, an extra bony "bridge" was noted to be present, intervening between two distal phalanges; the possible explanation for the latter structure is discussed below (section 8.5.6).

8.5.1.2 High incidence of postural defects in the limbs

Once the skeletal elements had been revealed, another unexpected finding was immediately obvious. With careful scoring, postural deformities involving the wrist and ankle regions (termed "clubhand" and "clubfoot" deformities, respectively) became more evident than in the previous study. This implied that the teratogenic effects of compression secondary to ASP on the fetal limbs was *underestimated* previously and should be reevaluated in the light of these further studies. Equally, the left side was more severely affected than the right side, suggesting an asymmetrical factor may play an important role when limbs were subjected to the compression force (see below).

8.5.1.3 Compressed pattern on the sternebrae

The most striking unexpected finding in this skeletal survey was the discovery of the compressed pattern of the sternebrae. In the treated embryos which displayed other defects, almost all of these fetuses (17 out of 19) had asymmetrically compressed sternebrae, with the left side often more severely affected than the right side. This observation was consistent with findings found in an earlier rat study (Love & Vickers, 1972). We thus proposed that both the developing sternebrae and limbs were subjected to the compression resulting from ASP, which was asymmetrically distributed due to the natural morphology of the embryos. During day 12 - 13 of gestation when the ASP procedure was normally performed, the tail of the embryo is directly slightly towards the right of the head; thus the head is somewhat facing to the left. With the compression following ASP, the head region immediately contacts the left limb, placing extra asymmetric force on the left limb. We also believe that this is the first time a reason has been suggested to explain the asymmetric effect following ASP. While the incidence of affected limbs was higher on the left side, neither intact bone lengths nor lengths of the ossification centres of the long bones of the two sides were formed to be different. The reason for the latter remains unclear.

8.5.2 Observation of a transitory fetal bradycardia

Ideally, after observing that the soft tissue of the distal limbs were particularly affected by ASP, the succeeding experiment was carried out to investigate this aspect in more detail. We noted that general ecchymoses and haemorrhages appeared all over the body -- including the head, trunk and

particularly in the distal limb region, confirming similar reports by others (Singh & Singh, 1973; Kennedy & Persaud, 1977; Houben, 1984). Accordingly, we decided to undertake an analysis of the heart rate of both the treated and non-treated "control" embryos, with the suspicion that the haemorrhagic lesions observed might be due to a *general* rather than *local* vascular stasis secondary to the compression following ASP.

8.5.2.1 Confirmation of the suspicion of vascular disruption

Results showed that a temporary bradycardia occurred in the "anaesthetic only" controls and the ASP-exposed embryos, with *an extra period of about 2 hours* (total 4 hours) observed in the ASP-exposed embryos. We believe that this is the first detailed study to demonstrate the general effect of fetal bradycardia and vascular stasis in the animal ASP experiments. Clinically, although a similar suspicion was proposed following CVS (Firth *et al.,* 1991b), no evidence of such a phenomenon has ever been demonstrated. We suspected that the reasons for the inability to demonstrate the bradycardia in clinical studies may have been due to several reasons: (i) low incidence of cases of amniontic rupture in the clinical studies (ii) small sample size being available in the clinical studies (iii) the analysis being performed *beyond the critical stage* when the craniofacial and limb abnormalities was suspected to be induced (iv) the occurrence of the fetal bradycardia being delayed rather than immediately after CVS, when the analyses were normally undertaken in these clinical studies (for details see section 4.4.3). By demonstrating fetal bradycardia following ASP, we confirmed the suspection that a general vascular stasis resulted from compression following ASP, and may play an important role in explaining the limb defects observed in both animal and clinical studies.

8.5.2.2 Avertin alone produces bradycardia

The most unexpected finding was that bradycardia of about 2 hours duration was also noted in the "anaesthetic only" control embryos. As normal morphology and very little haemorrhagic lesions were observed in this group, we proposed that the *extra* prolonged period of fetal bradycardia in the ASP-exposed embryos was critical and detrimental for the development of the distal limb. However, the influence of the compression of the extraembryonic membranes and the uterine muscles has to be considered as well. In the "anaesthetic only" control embryos, the bradycardia might remain as the only detrimental factor on the fetal limbs; no compression force was added at the same time. While in the ASP-treated embryos, the prolonged period of bradycardia, together with the compression from the extraembryonic membranes and the uterine muscles after ASP contributed equally to the abnormalities induced. After the fetal heart rate returned to normal levels, it remained unclear for how long the compression force would be in place.

8.5.3 Histological analysis of interdigital zones

Since we have revealed that the soft tissues particularly in the interdigital zones were the most affected area, and confirmed that a general vascular disruption occurred following ASP, we decided to investigate the underlying mechanism of how limb abnormalities were generated. In the majority of cases of adactyly and syndactyly in this model, no skeletal elements were missing or fused together, suggesting that the latter defects were due to the deficient separation of the soft tissues in the interdigital zones. We proposed that this might be either due to a *decreased* level apoptosis in the interdigital zones during normal limb morphogenesis, or due to an aberrantly *increased* cell proliferation rate in these areas. Histological analysis in the interdigital zones at various intervals after ASP was carried out in order to analyse the possible underlying mechanism(s).

8.5.3.1 Vascular disruption

Similar findings of vascular disruption as observed in the rat ASP studies (Love & Vickers, 1972; Singh & Singh, 1973; Kennedy & Persaud, 1977; Houben, 1984; Houben & Huygens, 1987) were also noted during the histological analysis of our material. Vascular congestion and haemorrhagic lesions were noticed in the area around the marginal vein and interdigital capillaries between 4 to 36 hours after ASP, suggesting that vascular disruption also existed in this mouse model and might therefore interfere with both the oxygen and nutrition supplies to the interdigital zones and consequently lead to the various abnormalities of this region observed in these mice.

8.5.3.2 Decreased apoptosis

Although no quantitative calculations could be undertaken due to the difficulty in recognizing individual apoptotic cells in the absence of specific stains for this purpose, the results nevertheless confirmed that the incidence of apoptosis was dramatically decreased in the interdigital zones in the ASP-treated embryos compared with that observed in the "internal" control embryos. We strongly suspected that this finding resulted from the experimentally induced vascular disruption as indicated above.

8.5.3.3 Increased mitosis

Using quantitative studies, we revealed that the mitotic activity of the interdigital mesenchymal cells dramatically *increased* in the ASP-exposed embryos, and that this was particularly evident between 4 to 8 hours after the ASP procedure. We proposed that this might be a compensatory reaction after the deprivation of the oxygen and nutrient supplies in the interdigital zones subsequent to the vascular disuption.

8.5.3.4 Diverted fates of the mesenchymal cells

The fate of the interdigital mesenchymal cells appeared to have been diverted following ASP. This was based on the observation that decreased evidence of apoptosis and increased evidence of mitosis was observed in the interdigital zones, as well as the occasional finding of an intervening skeletal "bridge" in this area between distal phalanges (Figure 3.2 d,e). All these results provided us with evidence of divergent pathways that the interdigital mesenchymal cells might adopt. We proposed that, within the undifferentiated interdigital mesenchymal cells, pre-set cellular programmes, which depend on their location and environment, might be interfered with by abnormal stimuli, e.g. following ASP. Similar findings were previouly observed in chick experiments when Janus Green B was adminstered into the amniotic cavity of developing chick embryos, instead of undergoing apoptosis in the interdigital zones, extra skeletal elements formed (Fallon, 1972; Fernandez-Teran & Hurle, 1984). In this mouse ASP model, the pre-determined apoptosis pathway of the interdigital mesenchymal cells was inhibited, possibly by the vascular disuption in these areas following ASP, and the development of these cells was altered, directing them to form skeletal elements or simply to continue to proliferate.

8.5.3.5 Future experiments for confirmation of apoptosis

One of the drawbacks in this particular study was that the extent of apoptosis in both the control and experimental groups could not be quantified. This was because of the uncertainity of quantification of the apoptotic bodies observed in the conventional paraffin histological sections. Should more resources, reagents and time been available, it is recommended that specific stains should be used to unequivocaly demonstrate the presence of apoptotic cells. For example, by applying either the fluorescent DNA-binding dye propidium iodide (or DAPI), or haematoxylin and eosin/toluidine blue, condensing chromatin in apoptotic cells could be identified more easily at the light microscopic level (Coucouvanis et al., 1995; Kerr et al., 1995).

8.5.4 Semi-thin section and genetic studies of activity in the interdigital zones

According to the results obtained from the previous histological analysis, it has been suspected that the vascular disruption may interfere with the pre-set programme which controls the cell fates in these areas; possibly due to alteration of certain genes in the vicinity. *Msx-1*, for example, was a favourable candidate not only because it is expressed in corresponding locations in the normal limbs, but also because the probe for this gene was readily available in our laboratory. Furthermore, this homeobox-containing transcription factor gene, *Msx-1*, is known to play a role in the maintenance of

proliferative and undifferentiated cells, as well as associate with the control of PCD (programmed cell death) in the interdigital zones (Akimenko *et al.,* 1995 and references therein).

Additionally, in the attempt to undertake the previous conventional paraffin histological study, a similar analysis using semi-thin sections was also employed.

8.5.4.1 Hypertrophied epitheium and overexpression of *msx-1* gene

Abnormal epithelium, particularly in the form of hypertrophy, and evidence of the overexpression of *msx-1* gene, was observed in the autopods in the ASP-treated embryos. The interrupted epithelium inevitably abolished the normal epithelium-mesenchymal interaction, which the normal expression pattern of *msx-1* gene largely depends on. In various transplant experiments (limb bud: Davidson *et al.,* 1991; Robert *et al.,* 1991; Haramis *et al.,* 1995; Kostakopoulou *et al.,* 1996, facial processes: Takahashi *et al.,* , 1991; Brown *et al.,* 1993, tooth buds: Jowett *et al.,* 1993, the epithelium of the perinatal Müllerian duct: Pavlova *et al.,* 1994), the expression of this gene was altered when the local environment of the epithelial-mesenchymal interaction was eradicated. This modification was suspected to induce the tissue regenerative activity in the interdigital zones, as *msx-1* has been strongly correlated with the regenerative ability on the distal part of the mouse limb (Reginelli *et al.,* 1995). We believed that this suspicion was confirmed by our previous findings of increased mitotic activity in the interdigital zones. Also, in the hypothetical role of *msx-1* gene in maintaining the interdigital mesenchymal cells in an undifferentiated and proliferative status, our findings showing both increased evidence of mitosis and overexpression of *msx-1* in these areas may provide additional evidence to confirm this hypothesis.

8.5.4.2 Future experiments for analysis of interdigital gene interaction

If more resources, reagents and time had been available, additional studies would have been initiated, to elucidate the underlying mechanisms that lead to soft tissue syndactyly following ASP with an attempt to investigate normal digit formation on a molecular basis. Transcripts of a considerable number of genes are found in the interdigital zones during limb development. *Msx-1* and *-2, BMP-2, -4,* and *-7, FGFR-1* and *-2*, and *Shh* are some genes that attract most attention recently (reviewed by Ferriti and Tickle, 1997). *Msx-1* has been shown to be overexpressed in the interdigital areas and interfered with the pre-set apoptotic and mitotic programmes of the interdigital mesenchymal tissues in this study. Similarly, *Msx-2* expression is altered following ASP and is suspected to be related to the soft tissue syndactyly (Chang and Kaufman, unpublished observations). Interestingly, *Msx-1* knockout mice exhibit no obvious abnormalities in the limbs (Satokata and Maas, 1994); probably due to functional compensation from *Msx-2* (Catron *et al.,* 1995; Catron *et al.,*1996). In contrast, *BMP-2/-4* have been proved to function in the chick limb through dominant-negative mutation of the

BMPR1 receptor, resulting in soft tissue syndactyly (Zou and Niswander, 1996). The results indicate that *BMP-2* and *-4* mediate interdigital apoptosis and may interact with *Msx-1* and *Msx-2* during normal digit formation. Thus, the mouse ASP model provides opportunities to elucidate the interaction of these genes and, at the same time, may help to further understand the molecular basis of digit formation.

8.5.5 Influence of various anaesthetic agents on the incidence and type of abnormalities observed following ASP

As indicated above, we believed that the abnormalities observed in the interdigital zones in our mouse model were due to a vascular disruption following ASP. Considering the observation that a temporary period of bradycardia was also found in the "anaesthetic only" control embryos, we suggested that various anaesthetic agents might alter the duration of bradycardia following the ASP procedure and subsequently interfere with the severity of the vascular disruption. An alternative anaesthetic agent, inhalational halothane, was then introduced to our mouse model in order to test the latter hypothesis. Compared with Avertin, which required 30-90 minutes for the operated females to fully recover, full recovery took only 3-5 minutes for the animals who were exposed to halothane as the anaesthetic agent. Repeated analysis identical to that decribed in chapter 2 was carried out using halothane instead of Avertin as the anaesthetic agent, and results from both studies were carefully compared. It was expected that with reduced post-operative recovery time, the survival rate might be improved and the incidence and type of the abnormalities observed would be different when compared to the previous study when Avertin had been used.

8.5.5.1 General findings

Findings from this study confirmed our suspicion. With the inhalational anaesthetic halothane, the survival rate of the ASP-treated embryos was improved by about 10%. While the incidence of cleft palate remained at an approximately similar level, the incidence and type of limb abnormalities observed was dramatically altered.

8.5.5.2 Decreased incidence of syndactyly

The incidence of syndactyly was found to be dramatically reduced. This, again, confirmed our suspicion indicated above that the cellular defect that results in syndactyly observed in the ASP mouse model was principlly due to the duration of the vascular disruption. This was based on the assumption that the inhalational anaesthetic agent, halothane, reduced the duration of the post-operative recovery period, we suspect that it probably also reduces the length of the temporary period of bradycardia, and consequently diminishes the vascular disruption following ASP.

8.5.5.3 Increased incidence of postural abnormalities

Unexpectedly, the incidence of postural abnormalities *increased significantly* in the current study, implying that the compression by the extraembryonic membranes and the uterine muscles was consistently observed following ASP, although the vascular disruption might be reduced to a considerable degree. However, we do not consider that the compression force generated by ASP was *greater* in the current study. The higher incidence of postural limb deformities may have been *underestimated* in the previous Avertin study, since a significantly *greater* incidence of clubhand and clubfoot was observed in the subsequent skeletal study (Chang *et al.*, 1996). Alternatively, the incidence of the postural limb deformity may not have been accurately determined as only gross morphology was evaluated in this current study, in the absence of "cleared" specimens. To reveal the skeletal elements appeared to be necessary if a proper quantitative analysis required.

8.5.5.4 Future experiments for the effect of Halothane in the mouse ASP model

In order to fully understand the reason why the incidence of syndactyly was reduced and postural deformity increased, an additional series of studies using halothane should be repeated identically as those carried out previously using Avertin. Should these had been performed, we believe that the duration of bradycardia would be reduced, and the behaviour of the interdigital mesenchymal cells and their genetic activity would inevitably have been altered. However, we believe that the incidence of postural limb deformities would probably be similar in both the Avertin and halothane experiments when "cleared" skeletal specimen were analysed.

8.6 Possible contribution to knowledge of limb development gained from the mouse ASP model

Although it has recently been recommended that CVS should not to be performed *before 9 weeks of gestation*, we appreciate that an animal model for studying the teratogenic effects generated during CVS is necessary. Using this model, we have been able to investigate the aetiology of those craniofacial and limb abnormalities observed, as well as indicating how similar abnormalities may be prevented in clinical practice by a greater understanding of the underlying mechanism of induction of these defects. We feel that a simple explanation such as "vascular disruption" as suggested in the earlier clinical studies is neither adequate nor satisfactory, and thus a suitable animal model should be used to expose the complicated mechanism(s) contributing to these abnormalities. Both the mothers and their infants born with craniofacial and limb abnormalities following CVS should be offered a rational explanation. Additionally, we believe that once a proper animal model is exclusively established and recorded in the literature, whenever a similar situation arises in clinical practice, an appropriate code of conduct could be followed. Had the results of the earlier animal studies on the

teratogenic effects of amniocentesis attracted more attention, a risk of the abnormalities following CVS performed before 9 weeks of gestation would almost certainly have been considered, and fewer genetically normal babies previously exposed to CVS would suffer from the OMLHS.

8.6.1 Important model for teratogenic studies

Apart from its significant role of acting as an animal model for investigating the abnormalities following ASP, we believe this is also an ideal model for both normal and teratogenic studies of digit formation. This is particularly interesting in the cases where the alteration of genetic activity and/or cell behaviour occurs following vascular disruption, hypoperfusion and/or tissue hypoxia. Modified experimental conditions can be studied by utilizing a wide range of anaesthetic agents.

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