PATHOGENESIS OF FETAL ABNORMALITIES INDUCED IN THE RAT BY AMNIOTIC SAC PUNCTURE

A Thesis

Presented to the Faculty of Graduate Studies,

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Master of Sciences

by

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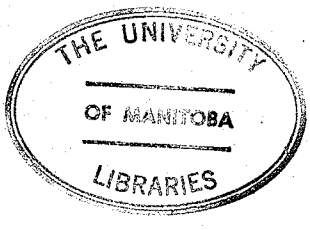

To my parents, and the many friends

who have offered their encouragement.

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ABSTRACT

The teratogenicity of experimental amniocentesis was investigated in the rat, and an attempt was made to reduce the deleterious effects observed. The incidence of fetal death was decreased by reducing the calibre of the needle used to make the puncture. Gestational age at the time of treatment and the volume of solvent vehicle injected were also found to influence the teratogenicity of amniotic sac puncture.

The morphogenesis of developmental defects induced by amniotic sac puncture was studied at the gross and microscopic levels. In fetuses recovered from 15 minutes to 48 hours after amniocentesis, a pattern of hemorrhagic lesions, excessive accumulation of interstitial fluid, followed by tissue necrosis and leading ultimately to the reduction or amputation of distal limb segments, was observed. These changes were indicative of venous stasis and embryonic oxygen deficiency. Intra-uterine compression of the fetus and the obstruction of the feto-maternal circulation were considered to be the primary aetiological factors in amniocentesis-induced anomalies which included hemorrhagic lesions, limb reductions and amputations, deformities of the head and abdominal regions, generalized oedema and postural moulding.

TABLE OF CONTENTS

SECTION PAGE

SECTION PAGE

STATEMENT OF THE PROBLEM

I. STATEMENT OF THE PROBLEM

In recent years investigators in the field of experimental mammalian teratology have attempted to treat the embryo directly with the suspected teratogen by injecting it into the amniotic sac. In doing so the mediating influences of the maternal organism and the placenta are eliminated without disrupting the physiological feto-maternal relationship or the integrity of the developing embryo. Still it is not clear whether the results of such studies are due exclusively to the test substance or if in fact, the experimental procedure itself is not at least partially responsible.

The objectives of the present investigation are as follows:

a) to determine the effects of puncturing the amniotic sac on the developmental processes of the rat;

b) to investigate the pathogenesis of lesions induced by puncturing the amniotic sac;

c) to assess the usefulness of the intra-amniotic route of treatment in teratological studies;

d) and to attempt, because of procedural similarities, to relate this study to the clinical aspects of amniocentesis.

SECTIONS I - 3

REVIEW OF RELATED LITERATURE

2. REVIEW OF RELATED LITERATURE

2.1 THE AMNIOTIC MEMBRANES.

The membranes surrounding the human amniotic cavity, namely the amnion, chorion and initially the decidua, evolve from both maternal and embryonic tissues in response to the nidation of the blastocyst. Various functions have been attributed to these membranes including protection, nutrition, respiration, excretion, and the formation and circulation of the amniotic fluid (Taussig, 1927; Plentl, 1961; Bourne, 1962; Saunders and Rhodes, 1973). The control of the volume of the liquor amnii (McInroy and Kelsey, 1954; Scott and Bain, 1958; Jeffcoate and Scott, 1959; Bourne, 1962; Behrman, Parker and DeLannoy, 1967; Abramovich, 1973) and the maintenance of a fluid environment for the fetus have also been listed as functions.

Many abnormalities of growth and development have been associated with abnormalities of the amniotic membranes and amniotic fluid volume (Jeffcoate and Scott, 1959; Hertig, 1960; Bourne, 1962; Patterson, 1962; Torpin, 1965), both of which play an important role in the normal development of the offspring.

2.1.1 The Derivation of the Amniochorionic Membrane

The extra-embryonic or fetal membranes which are derived from the zygote are the amnion, the chorion, the yolk sac and the allantois. Only the allantois and the yolk sac are concerned with the formation of embryonic structures.

The amnion, the innermost layer of the amniochorionic membrane, is derived from amniogenic cells in the cytotrophoblast and initially consists only of epithelium and the extra-embryonic somatopleure (Bourne, 1962). The amniotic cavity forms by the coalescence of small spaces which form between the invading trophoblast and the embryoblast. The amnion, forming the roof of this cavity, is continuous peripherally with the floor of

the cavity, the embryonic ectoderm. Following longitudinal and transverse folding early in the embryonic period, the amnion also provides the epithelial sheath of the umbilical cord.

The chorion evolves from the extra-embryonic somatic mesoderm and from cells which differentiate from the cytotrophoblast. The large chorionic cavity surrounding the embryo, its amnion and yolk sac, forms by the coalescence of fluid-filled coelomic spaces within the proliferating extra-embryonic mesoderm. Initially villi cover the entire surface of the chorion but with the enlargement of the sac into the uterine cavity, villi remain only on the chorion frondosum which constitutes the fetal component of the placenta. The remaining chorion laeve is bare.

The amniochorionic membrane is formed by the fusion of the amnion and the chorion. This fusion obliterates the chorionic cavity. Further enlargement of the amniotic cavity results in the fusion of the amnio-chorionic membrane with the decidua capsularis. The decidua soon attenuates and degenerates. With further amniotic cavity expansion, the amniochorionic membrane fuses with the decidua parietalis. In doing so, the true uterine cavity is obliterated and the sac of pregnancy, bounded by a membrane of mixed origin occupies the entire uterine space. This composite membrane is referred to as the amniotic membrane(s) or fetal membranes. The blood vessels of these fetal membranes as a rule atrophy and disappear completely as pregnancy progresses (Bourne, 1962).

2.1.2 The Anatomy of the Amniotic Membranes

The microscopic and ultrastructural features of the human amnion and chorion have been reviewed in detail by Mossman and Noer (1947),

Dempsey and Wislocki (1953), Boyd and Hughes (1954), Wislocki and Dempsey (1955), Bourne, (1960, 1962) and Bergstrom (1971).

2.1.2.1 The amnion

The embryonic amnion consists of two layers, the epithelium and the extra-embryonic somatopleure. The amnion differentiates further into the five-layered structure which is found at term.

The amnion is the innermost layer of the amniochorionic membrane. It lines the fetal aspect of the amniotic cavity and is in immediate contact with its contents, namely the liquor amnii and the fetus (Bourne, 1962). There is a placental portion and a portion forming the epithelial sheath of the umbilical cord which is continuous with the fetal epidermis. At term the amnion has no blood supply and in early pregnancy the presence of a blood supply is disputed.

Microscopic and ultrastructural studies of the amnion indicate that it is a complex, functional tissue composed of the following five layers:

The epithelial lining is typically a single layer of cuboidal cells. However, flattened cells are often found in the reflected amnion and columnar cells are frequently present on the placental amnion. The apex of the cells present small evaginations of cell membrane protruding from the surface forming a brush border. Basally there are irregular cytoplasmic extensions which are in intimate contact with the underlying basement membrane (Danforth and Hull, 1958). In the cytoplasm, vacuoles

of varying size, number, location and content have been reported. Many hypotheses have been put forward regarding those vacuoles but their functional significance remains unsettled. A secretory activity has been attributed to them (Danforth and Hull, 1958), they have been considered to be artefacts (Schröder, 1955, cited in Bourne, 1962) and they have been implicated in the formation of the vernix caseosa (Keiffer, 1926, cited in Bourne, 1960).

Examined at higher magnification the vacuoles are found to consist of distended portions of a complex intracellular canal system. Ultrastructural studies show evidence of surface microvilli, basal processes, lateral and basal vacuoles and an extensive system of canals and channels which communicate directly with the extra-cellular space (Bourne, 1962).

The basement membrane is a narrow band of reticular tissue lying along the base of the epithelial cells to which it is securely attached.

The compact layer is an acellular layer composed mainly of reticular fibres embedded in a connective tissue matrix of similar density whose resistance to leukocytic infiltration is clearly demonstrated in the presence of a severe inflammatory response within the membranes (Bourne, 1962).

The fibroblast layer is a complex reticular network secreted by the fibroblasts which are distributed diffusely throughout the network. The fine structure of the fibroblasts is variable, perhaps related to age, physiological or pathological states. The relatively scarce Hofbauer cells present in this layer are macrophages.

The spongy layer is the fifth and outermost layer and is composed of extra-embryonic coelomic tissue which has been compressed between the chorion and the enlarging amniotic sac. This intermediate layer adheres to both membranes but adhesion to the amnion is more pronounced. It varies greatly in thickness and consists of an intricate, fine fibrous network bathed in mucus which is very hygroscopic and will become thick, distensible, and occlementous, thus facilitating a considerable degree of movement of the amnion upon the underlying, fixed chorion. It may also act as a selective barrier for substances passing between mother and fetus. Fibroblasts and Hofbauer cells are present.

2.1.2.2 The chorion

The human chorion is interposed between the maternal decidua capsularis and the fetal amnion. At twelve weeks gestation, when the true uterine cavity is obliterated, the thin attenuated capsularis separates the chorion from the decidua parietalis.

The embryonic chorion is a two-layered villous structure, one layer being the extra-embryonic somatic mesoderm and the other being a layer of differentiated cytotrophoblastic cells. With further differentiation a four-layered mature chorion evolves.

The mature chorion has a villous portion called the chorion frondosum which is the fetal component of the placenta, and a smooth chorion laeve, whose villi degenerate as the chorionic cavity enlarges into the uterus. The portion related to the internal os of the cervix is known as the dependent chorion.

The blood vessels occupying the villi of the early chorion atrophy and degenerate. The only remaining vascular channels are localized in the chorion frondosum. There is no evidence of a capillary bed for its own nutrition.

Starting on the inner or amniotic surface of the chorion laeve the four component layers are as follows:

The cellular layer, which is frequently absent in the term chorion, is similar to the fibroblast layer of the amnion. It is a thin network of interlacing fibroblasts.

The reticular layer contains fibroblasts and Hofbauer cells in a reticular network, the fibres of which penetrate deeply into the trophoblast binding the different layers of the chorion together.

The pseudo-basement membrane is seen as a narrow acidophilic band of reticular tissue forming the basement of the trophoblast. The prefix "pseudo" is used to distinguish it from the basement membrane of the amnion.

The trophoblast, is an irregular layer of trophoblast cells ranging in depth from two to ten cells, immediately adjacent to the maternal decidua basalis and capsularis. The cytotrophoblast seen early in pregnancy is normally not found at term. Atrophic or ghost chorionic villi and necrotic plaques are seen in the form of fibrinous deposits in the trophoblast (Bourne, 1962).

2.1.3 The Pathology of the Amniotic Membranes

Abnormalities of the amnion are often associated with congenital malformations. Abnormalities of the membranes include epithelial abnormalities, premature rupture, amnion nodosum, squamous metaplasia, inflammation of the membranes and cord and amniotic bands (Bourne, 1962). A brief discussion of abnormalities relating to this work follows.

2.1.3.1 Amnion nodosum

Landing (1950) coined the term "amnion nodosum" after reporting eight cases in which a pathological condition of the amnion characterized by multiple greyish nodules studding the fetal surface of the amnion was described. This was apparently associated with a deficiency in the secretion of fetal urine. Von Franque (cited in Bourne, 1962) reported the same phenomenon as "Amnionknotchen" in 1897. Further study of this condition in cases where amnion nodosum and oligohydramnios existed in the absence of fetal renal agenesis, dysplasia or urinary obstruction led Jeffcoate and Scott (1959) to conclude that the primary association of amnion nodosum was with oligohydramnios or anhydramnios and only secondarily related to a deficiency of fetal urine secretion.

The lesions characteristic of amnion nodosum are small, dull grey-yellow to brick-red plaques ranging in diameter from 0.1 mm to 4.0 mm. They are found on the fetal surface of the amnion and umbilical cord, concentrating and most obvious on and near the placental amnion. They can be picked off the amnion without interrupting its continuity and they move with the amnion as it slides on the chorion. Scott and Bain (1958) described the nodules histologically as "masses of keratinized squames embedded in an amorphous acidophil matrix." Jeffcoate and Scott (1959) described them as "disorganized masses of keratinized squames of fetal epidermis loosely embedded in the amnion". The cuboidal cells typical of the amnion are generally absent ruling out the possibility of squamous metaplasia as an explanation.

Bourne (1962) presents a theory of the aetiology of amnion nodosum in which the nodules are the result of friction between fetal epidermis and the amnion epithelium. This would occur in cases where there is a deficiency of amniotic fluid and a concomitant reduction in the size of the amniotic sac. The friction causes the shedding of the fragile fetal epidermis with the destruction of the cuboidal amnion epithelium caused either by the mechanical friction or the deposition and accumulation of these squames. Whatever the correct aetiology, amnion nodosum is a pathological condition of the amnionic surface which is specifically associated with oligohydramnios or anhydramnios.

2.1.3.2 The amniotic band syndrome

The amniotic band syndrome has been known for at least 300 years and was believed to be of mechanical causation. These amniogenic lesions, first described by Chaussier in 1812, are distinct from similar lesions of genetic causation in that they tend to be a) multiple but not bilaterally symmetrical, b) associated with constricting bands and c) associated with clubbing of the feet. These three conditions often present a pathognomonic triad as described by Torpin (1965).

In 1930, Streeter disputed the mechanical theory of causation and suggested that the ring-constriction lesions were localized areas of imperfectly formed tissues resulting from defective areas of germ plasm. The fibrous bands frequently associated with this syndrome were believed to be part of the lesion itself and to represent abnormal desquamated tissue. Patterson (1959, 1961, 1962) supported this "failure of development" theory.

Torpin (1965) and Browne, (1955) supported the mechanical theory which they substantiated with numerous case studies. It was proposed that the fibrous strings were formed by the degeneration and rolling up of the amnion following its premature rupture, and themselves caused the ring-constrictions and amputations. Loss of the amniotic fluid through the denuded chorionic surface would result in a temporary oligohydramnios which could cause the clubbing of the feet commonly associated with ring-constrictions.

The free-floating amnion or fibrous bands threaten the fetus and umbilical cord with entanglement, with results ranging from ring-constrictions with or without ulceration, amputation of a portion of the fetus, death by strangulation to abortion.

2.2 THE AMNIOTIC FLUID

Hippocrates postulated the oldest, most persistent and simplest theory that the liquor amnii was the product of the fetal kidneys. In later times, it was considered to be an inert, stagnant pool surrounding the fetus. In a comprehensive review of the subject, Shaw and Marriott (1949) revealed that few theories have stood the test of time. Recent investigations using radioactive isotope tracer techniques (Flexnor et al., 1948, Vosburgh et al., 1948; Plentl and Gray, 1954, 1957; Hutchinson et al., 1959) have shown, however, that a) amniotic fluid has a complex cellular and chemical composition which is in a continual state of flux, b) that its formation, circulation and

disposal represent many dynamic and finely balanced processes similar to other fluid compartments of the body, and c) that deviations from the normal volume and composition constitute a threat to the development of the fetus.

2.2.1 The Origin of the Amniotic Fluid

2.2.1.1 The role of the amniotic membranes

The fact that a) amniotic fluid is present before the fetal systems are well-differentiated and functional, b) fluid is often present in the sacs of blighted, rudimentary or absent ova, c) the composition of the liquor of early pregnancy is similar to the maternal plasma, and d) the presence of secretory cells in the amniotic epithelium early in pregnancy (Saunders and Rhodes, 1973) imply that at least in early pregnancy, the amniotic fluid enters the sac either actively or passively via the amnion. Since the maximum rate of increase in fluid volume corresponds to the time when amniotic cells degenerate and since polyhydramnios is associated with the complete absence of secretory cells (Plentl, 1957) it seems likely that the role of the amnion is active rather than passive. However, more histological and ultrastructural evidence supporting the active secretory role of the amnion is required before this can be considered conclusive (Bondi, 1905, cited in Bourne, 1962; Taussig, 1927; Danforth and Hull, 1958; Bourne and Lacy, 1960).

Whereas the relatively simple epithelial cells of the early amnion would facilitate the passive passage of a maternal transudate, the development of microvilli and a complex network of intracellular and

intercellular canals in later pregnancy suggest that at this time the movement of material is highly specialized and selective (Bourne and Lacy, 1960). It is unlikely that the connective tissue layers of either the amnion or chorion would resist the passage of fluid from the intervillous spaces of the placenta or the decidual vessels, to the epithelial layer of the amnion (Bourne, 1962).

The placental amnion particularly, by virtue of its vast vascular connections and columnar epithelium (suggestive of an increased functional state), has been implicated in the production of amniotic fluid. Theoretically this is possible (Saunders and Rhodes, 1973) but more evidence would be necessary to attribute this function exclusively to the placental amnion (Bourne, 1962).

On the basis of the morphology of the term amnion, Bourne and Lacy (1960) proposed a working hypothesis:

(a) The fluid enters the amnion via the spaces between adjacent cells where it passes into the lateral system of vacuoles;

(b) fluid also enters via the apical aspect of the cell whose surface area is greatly increased by the presence of microvilli;

(c) once in the cell, the fluid enters the complex system of fine canals and channels which communicate with the lateral and basal vacuoles and hence with the intracellular space. This results in an enormous increase in surface area. Within the vacuoles there is probably some mixing of solutions entering via the two pathways;

(d) assuming that different ions travel more readily along one route than the other, a much over-simplified explanation is possible for the differential rate of turnover of the different ions;

(e) having permeated the epithelium, only the connective tissue layer, consisting mainly of a fibre network, remains; little obstruction at this point likely, at least on morphological grounds.

From their studies with monkeys, Behrman et al. (1967) postulated that in early pregnancy the amniotic fluid is a transudate of maternal plasma. It becomes more fetal in composition only in later pregnancy as the fetus contributes in increasing amount of hypotonic urine and other body secretions to the amniotic space. This was supported by the fact that when the fetus was removed from the sac the composition of the fluid remained similar to maternal plasma. The development of a stratified squamous, keratinizing epithelium, vernix caseosa and lanugo hair further separate the two fluid compartments and influence the differing composition of amniotic fluid and maternal plasma late in gestation.

2.2.1.2 The role of the fetus

Theoretically the fetus could excrete fluid from its renal tract, respiratory tract or integumentary system and appendages (Saunders and Rhodes, 1973). It is possible that the fetus contributes to the volume and composition of amniotic fluid only in late pregnancy. It is generally accepted that the fetal kidneys become functional by week 20 of gestation (Bourne, 1962, Saunders and Rhodes, 1973) and that by late pregnancy, contribute up to 500 ml. of urine per day to the liquor (Moore, 1973). Keratinization of the fetal epidermis essentially separates the two fluid compartments physiologically. These two factors

likely explain the changes in the chemical composition of the liquor which commence in mid-pregnancy, such as the increase in urea, uric acid and creatinine in both amniotic fluid and fetal urine. No direct evidence has been found to show that the salivary glands, buccal mucosa, lungs, trachea or skin of the fetus contribute significantly to the amniotic fluid volume (Saunders and Rhodes, 1973).

Plentl (1961) has shown that the significance of the umbilical cord to the amniotic fluid lies not in the actual formation, but rather in the transportation and exchange of the fluid between the amniotic cavity and the fetus.

2.2.2 Circulation and Disposal of the Amniotic Fluid

2.2.2.1 Umbilical cord

Hutchinson et al. (1959) demonstrated that 30 and 120 minutes after the injection of radioactive tracers into the amniotic fluid fetal urine had 30% and 40% respectively of the cord blood levels. Wharton's jelly had an isotope concentration several times higher than that of cord blood but lower than that of amniotic fluid at the time of termination. This gradient of diminishing concentrations from amniotic fluid to Wharton's jelly to umbilical cord blood implies that the isotope passes directly across the cord epithelium in relatively large amounts.

2.2.2.2 Amniotic membranes

Although it is not certain exactly how many of the various components can be transported nor at what speed, the movement of

materials and fluid across the membranes from the amniotic cavity directly to the maternal circulation has been demonstrated to occur (Bourne, 1962).

2.2.2.3 Fetal ectoderm

Fetal skin and the respiratory tract may absorb water early in pregnancy but likely in very small amounts. The finding of hair, vernix caseosa and epithelial cell debris in the fetal intestine, in conjunction with the staining of the intestinal tract by dyes injected into the amniotic sac, implicate the alimentary tract as the major fetal pathway for the disposal of amniotic fluid. Gastrograms of radio-opaque material proves that not only does the fetus swallow amniotic fluid but also that the stomach absorbs some of it (Jeffcoate and Scott, 1959).

Gray et al. (1956) estimated, by the use of isotopes, that at least 25% and probably more than 50% of water transport from the amniotic sac to the mother is accomplished through the fetus. As pregnancy progresses, the role of the fetus in the transfer of amniotic fluid becomes more significant such that by term, at least 40% of the water transfer to the mother is accomplished via the fetus (Bourne, 1962).

Morphological changes in the fetal epidermis as pregnancy progresses suggest that it is involved in the exchange of water and electrolytes between the amniotic fluid and fetus. At 8 weeks the fetal epidermis is composed of a basal layer and a superficial layer of cells or periderm. At 3 months intermediate cell layers appear and by the

4th month "bladder cells" form a second layer of globular peridermal cells on the epidermal surface. At this stage the peridermal cells appear to be functionally active, with surface microvilli, Golgi apparatus, mitochondria, ribosomes and endoplasmic reticulum. By 17-20 weeks the fetal periderm begins to disappear and the underlying epithelium begins to keratinize (Hoyes, 1968).

2.2.3 The Regulation of the Amniotic Fluid Volume

The factors regulating the volume of amniotic fluid are closely linked with the circulation of the amniotic fluid and the water dynamics of the gestational sac. The factors controlling the volume of the amniotic fluid are largely unknown, the importance of fetal swallowing and voiding is disputed, and the part played by the exchange of water and electrolytes between the fluid, fetus and mother is ill-understood (Abramovich, 1973).

The close relationship between the amniotic fluid volume and the fetal surface area, and the fact that the cells of the periderm, prior to their keratinization, bear a striking resemblance to renal tubular cells under the influence of vasopressin, led Lind and Hytten (1970) to believe that the fetus exerts a controlling influence over the amniotic fluid volume via the fetal skin and that at least up to mid-pregnancy amniotic fluid is an extension of the fetal extracellular fluid space.

The results of the investigations by Parmley and Seeds (1970) into the fetal skin's permeability to isotope water, support the hypothesis that early in pregnancy fetal skin may serve as a pathway of water exchange between amniotic fluid and fetus. With the keratinization

of the fetal skin at about 18 cm CR length the permeability of the fetal skin changed from values approximately equivalent to the amnion and chorion laeve, and demonstrated a marked decrease in its diffusion constant. In well-keratinized skin there was no appreciable diffusion.

Abramovich (1973) made the following conclusions from the results of in vivo studies of human fetal water transport:

i. As the fetal size increased the amount of isotopic water absorbed via the skin was reduced;

ii. Up to 18 weeks of gestation, the cord played no part in water absorption whereas after 18 weeks the cord began to absorb water.

It seems likely then, that when keratinization of the fetal skin begins between 17 to 20 weeks, the unkeratinized epithelium of the umbilical cord takes over the function of transporting water from the amniotic fluid to the fetus. This is supported by evidence presented by Hutchinson et al. (1959) which demonstrated a high rate of transfer of isotopic water through the umbilical cord at term.

2.2.4 Composition of the Amniotic Fluid

The normal constituents of the amniotic fluid, their normal values, and the association of deviations from these norms with pathological states of the fetus have recently been the subject of extensive investigation and are recognized to be of great clinical value.

2.2.4.1 Water

Seeds (1968) stated that a steady state exchange of water and electrolytes between the intra-uterine compartments takes place involving fetal swallowing, urinary excretion and the other significant

exchanges of solute and water according to the physicochemical principles already described *in vitro* for these tissue layers.

2.2.4.2 Biochemical composition

Changing concentrations of sodium, urea, creatinine and chloride throughout gestation have been studied by Lind (1973). Räihä (1963) reported levels of organic acids such as pyruvic, αketoglutaric and citric acid. Schreiner (1967) has published levels for glucose and lactic acid.

In early pregnancy, Lind (1973) pointed out that the concentrations of the more diffusible solutes such as sodium, chloride and urea in the amniotic fluid are closer to concentrations of fetal serum than maternal. In the second half of pregnancy the amniotic fluid ceases to resemble any other fluid compartment and appears to be most influenced by the increasing contribution of fetal urine.

The biochemical and cytological analysis of amniotic fluid is capable of yielding valuable information regarding the functional and gestational maturity of the fetus (Lind and Billewicz, 1971).

2.2.4.3 Cellular constituents

The two most important variables affecting the cellular constituents of the amniotic fluid are the gestational age and the sex of the fetus. Prior to the 19th week of pregnancy both Huisjes (1968) and Wachtel et al. (1969) were able to recover very few cells from the fluid. With the advance of pregnancy a rapid rise in the number and the morphological cell types have been reported (Blysted et al., 1951; Votta et al., 1968; Huisjes, 1968, 1970). Huisjes (1973) traced the origins of the various epithelial cell types found in the amniotic fluid and, on the basis of morphological and histochemical criteria,

grouped the cells according to the following sites of origin: fetal epidermis, amnion (including the placental, reflected and umbilical portions), fetal digestive and respiratory mucosa, and urogenital epithelia.

The clinical applications of amniotic fluid cytology include assessment of gestational age, fetal sex, blood group and genetic disposition. The identification of fetal epidermal cells in the vagina is also the most reliable tool in the diagnosis of ruptured membranes (Huisjes, 1973).

2.2.4.4 Proteins and amnion acids

Extensive work has been done on the changing levels of the total protein content and the individual protein and amino acid levels with increasing gestational age (Queenan, 1973). Trends have been identified, deviations from which are related to various fetal disease states and which therefore, have considerable clinical significance. Perhaps the most important change in total protein levels occurs in erythroblastosis fetalis (Halitsky and Krumholtz, 1970). Also there are suggestions that fetal growth is retarded when amino acids available to the fetus (presumably as reflected in the amniotic fluid) are diminished.

2.2.4.5 Hormones

Investigation into the levels of hormones or their metabolites in the amniotic fluid has been stimulated by the hope that they may reflect fetal or placental condition. Such hormones as the estrogens, cortisone, cortisol, pregnanediol, human chorionic gonadotrophin (HCG), human placental lactogen (HPL), human chorionic

somatomammotropin (HCS), pituitary prolactin, renin and prostaglandins have been assayed. Although the clinical value of these measurements remains to be established, considerable information has been accumulated on the mechanisms by which amniotic fluid levels of hormones are maintained (Josimovich, 1973).

2.2.4.6 Enzymes

It has been known for well over 40 years that enzymes are detectable in the amniotic fluid. Recently the study of amniotic fluid enzymes has provided valuable insight into the interaction of the fetus with its environment. It has provided a valuable tool for the prenatal diagnosis of genetic disorders such as inborn errors of lipid, carbohydrate, mucopolysaccharide and amino acid metabolism (Nadler and Burton, 1973).

2.2.5 The Pathology of Amniotic Fluid Volume

Although the amniotic fluid and its constituents are in a continual state of flux throughout pregnancy the volume of liquor remains fairly constant for each particular stage of pregnancy. A normal volume of fluid is known to be of great importance in providing a favourable environment for the developing fetus. The normal volume and the pathological deviations from this are likely determined by the relative efficiency of the mechanisms for production, disposal and circulation of the amniotic fluid.

2.2.5.1 Liquor volume and fetal weight

In a survey of 50 cases of renal agenesis or dysplasia, Scott and Bain (1958) found that oligohydramnios or anhydramnios was definitely involved in 35 of these and was likely a feature in the majority of the others. Harrison and Malpas (1953) also found a correlation between liquor volume and fetal size. These investigators agreed that the normal increase in fluid volume acts as a stimulus for continued uterine distension. This anticipates fetal growth and allows both adequate space and good placental circulation upon which normal development is dependent. The fetus which fails to urinate in the last half of pregnancy becomes stunted due to the concomitant oligohydramnios. This happens only from the 34th week on implying that only then are the kidneys a major contributor to amniotic fluid volume.

Jeffcoate and Scott (1959) recognized the association between oligohydramnios and dwarfing, also the relationship between polyhydramnios and excessive development. They suggested that the fluid volume plays a facultative role, either limiting or allowing fetal growth.

2.2.5.2 Placental size, liquor volume and fetal weight

Placental insufficiency has been implicated in oligohydramnios although the exact mechanism is unknown. It is presumed that placental growth beyond mid-pregnancy occurs pari-passu with the portion of the uterine wall to which it is attached. Thus with greater uterine

distension a larger placenta develops (Jeffcoate and Scott, 1959). This may explain the relationship between fetal size, fluid volume and placental size but does not define a causal mechanism where abnormalities exist.

2.2.5.3 "The moulding hypothesis"

Browne (1955) discussed a poorly acknowledged class of deformities produced by mechanical faults during the process of gestation. He identifies three physical mechanisms by which the developing fetus may be malformed:

a) Malposition or the prolonged folding of the fetus in an abnormal position. This facilitates changes in joint mobility and in both hard and soft tissue alignment.

b) Increased mechanical pressure on a fetus before birth, due for example to a smaller maternal abdomen, should have similar effects to a plaster cast applied too lightly to a limb in post-natal life. This is substantiated by the atrophied muscles and stiff joints observed in children emerging from small pregnancies with a history of intra-abdominal pressure. The legs and feet are more affected than are the smaller arms and hands which are sheltered by the overhanging fetal head.

c) An increased hydraulic pressure or an increase in the tension of the amniotic fluid caused either by hydramnios or a small abdominal cavity, might be expected to interfere with the venous return from the limbs. According to the laws of hydraulics such pressure would

be equally distributed on all four limbs and the effects more severe with increasing distance from the fetal heart. This is confirmed by the reports of maternal discomfort in the histories of babies born with arthrogryposis and in babies with limbs and joints stiffly extended emerging from hydramniotic pregnancies.

Dunn (1974) also discussed congenital postural deformities associated with intra-uterine moulding of a previously normally formed part. He described three further factors, the rate of fetal growth, fetal plasticity and fetal mobility, which also affect the incidence of congenital malformation.

2.2.5.4 Arthrogryposis multiplex congenita (AMC)

Estimates of the prevalence of congenital club foot or talipes range from 8-139 per 10,000 births. (McIntosh et al., 1954; Davis, 1957; Ivy, 1957; U.N. Scientific Committee on the Effects of Atomic Radiation, 1958). The highest figure is based on a prospective study of over 5000 pregnancies (McIntosh et al., 1954) and probably reflects the most accurate estimate.

Drachman and Coulombre (1962) studied the pathogenesis of experimentally-induced AMC by immobilizing chick embryos with varying doses of curare infused for varying periods of time. Since there was no evidence of spinal cord or muscle abnormalities, since control infusions with the solvent vehicle had no detrimental effects and since, in general, higher concentrations of curare and more prolonged infusion produced more severe and widespread joint involvement, it

was concluded that immobilization produced the results seen and that the final fixed posture of the limbs was related to the curarized embryo's position in the shell.

Movement *in utero* is considered to play an important role in the development of the joints. Joint differentiation proceeds to a considerable extent in the absence of movement but articular cavity formation and the fine sculpturing of the cartilagenous surfaces require the mechanical action normally provided by the movement of the fetal skeletal system (Drachman and Coulombre, 1962). Congenital malformation evolving from mechanical errors, as discussed by Browne, (1955, 1967), the many joint deformities associated with disorders of the nervous and muscular systems and those associated with abnormalities of amniotic fluid volume are aetiologically distinct but have in common some degree of pre-natal immobilization.

2.2.5.5 Oligohydramnios and polyhydramnios

Oligohydramnios and anhydramnios are recorded clinically far less frequently than polyhydramnios, perhaps because it is difficult to diagnose and tends to go unnoticed. It is significant primarily in the last half of pregnancy.

Jeffcoate and Scott (1959) listed the causes of oligohydramnios as bilateral renal agenesis, urethral obstruction, post-maturity and retention of a dead fetus in utero. Bilateral renal agenesis is typically associated with pulmonary hypoplasia and death is usually attributed to asphyxia. A history of threatened abortion in the early months of pregnancy is also associated with oligohydramnios. In

such instances large areas of the maternal aspect of the membranes have been found at delivery to be covered with partially organized blood clot. This could have interfered with the amnion's contribution to the amniotic fluid volume.

In a review of 169 consecutive cases of hydramnios encountered in the practice of one hospital, Jeffcoate and Scott (1959) revealed that in 54 (32%) the excessive amount of liquor was associated with a malformation which would probably interfere with the fetus' swallowing or absorbing of amniotic fluid from the intestine such as anencephaly, iniencephaly, hydrops foetalis with gross oedema of the fauces, oesophageal atresia, duodenal atresia and diaphragmatic hernia. Statistical correlation indicated a causal relationship in hydramnios with maternal diseases such as diabetes mellitus, toxemia of pregnancy and Rh-incompatibility, congenital malformations of the fetus and multiple pregnancies. These have as a common denominator a pathologic metabolic or mechanical change which might interfere with the feto-maternal circulation (Plentl and Gray, 1957). Physicochemical investigations of water exchange cited previously have definitely implicated this circulatory system as a primary aetiological factor.

Experimental evidence supporting the association of abnormal volumes of amniotic fluid with congenital malformations was provided by Gulienetti et al. (1962). Using the radioisotope dilution method the amniotic fluid volume of individual control and teratogen-treated rats and mice were measured. Two substances of known teratogenicity

were used - a riboflavin-deficient diet and sodium salicylate. Although the absolute volume of amniotic fluid was not significantly different between the control fetuses and the normal offspring of treated females, the latter were generally smaller. Thus the difference between fluid volumes relative to fetal body weight suggested an increase in amniotic fluid in the normal young of treated rats. In the affected young of treated females the absolute volume of liquor was significantly increased. The trend towards an increase of liquor volume in cases of existing fetal malformation is evident.

2.3 AMNIOCENTESIS

2.3.1 Experimental Puncture of the Amniotic Sac

As early as 1956 Trasler et al. reported the teratogenicity of puncturing the amniotic membranes of fetal mice. She reported a high incidence of abortion and resorption, as well as cleft palate in 10 out of the 17 treated survivors. These results were attributed to the loss of amniotic fluid which resulted in compression of the fetus by the membranes. Since that time several papers have appeared referring to this procedure as experimental oligohydramnios (Persaud, 1973), experimental amniocentesis (Singh, 1973, 1974) and the intra-amniotic injection method (Dostal, 1971, 1973).

Kendrick and Feild (1967) confirmed the observation of Trasler et al. and, by performing amniocentesis on adrenalectomized rats, demonstrated that the malformations were not attributable to a maternal stress response mediated by the corticosteroids.

Singh et al. (1974) performed amniocentesis on Wistar rats at day 15 of gestation and at the same time withdrew 0.04 ml of amniotic fluid. They reported a fetal mortality rate of 56% and, of the treated survivors 75% had one or more malformations including palatal defects, exencephaly or encephalocoele, hydrocephalus, spina bifida, stunting and shorter umbilical cords. Limb malformations detected in the offspring included varying degrees of agenesis (micromelia, adactyly), malrotations, constriction rings, amputations and hematomas.

In another study, De Meyer and Baird (1969) found that amniocentesis-related death and malformations were age-dependent. The earliest treatment on day 14.5 of gestation was associated with the highest, and treatment on day 16.5 with the lowest mortality and malformation rates. Malformations such as small, thick trunks and necks, club feet, primitive digits or adactyly, scoliotic tails, microstomia and short umbilical cords were observed in the experimental fetuses but not in the controls. The causal mechanism was postulated to be oligohydramnios and intra-uterine immobility.

In 1971 Poswillo and Sopher suggested that hypervolaemia or hypoxia, by leading to fetal hypertension, was involved in amniocentesis-induced anomalies.

Love and Vickers (1972) studied the morphology of these lesions in an attempt to define a causal mechanism. They reported specifically on the dysmelia which is commonly associated with the amniocentesis syndrome in rats. Almost all limb defects were of the reduction type with defect intensity ranging from longitudinal splitting of the phalanges and phalangeal suppression, to severe growth retardation

of the long limb bones and limb girdles. A strain-specific effect was ruled out by using both Wistar and Sprague-Dawley rats and finding that the lesions of both strains were indistinguishable. The total limb defects were regarded to be a combination of a) retarded growth in the long bones, b) arrested or aberrant differentiation of the bones in the digital rays, and c) destruction of digital structure by hemorrhage and possibly localized ischemic tissue necrosis. Among the associated defects reported were cleft palate, anomalies of the abdominal wall, genitourinary tract defects and short umbilical cords.

In 1972 Persaud reported a high incidence of meromelia and stunted offspring in oligohydramnios induced by membrane puncture in pregnant Wistar rats. Since the embryological primordia of the limbs are established by day 14 of gestation these findings were taken to indicate that degenerative changes had occurred at the apical region of the limb buds, probably due to the restrictive and compressive effects of the fetal membranes and uterine wall on the apical primordia and their blood vessels. The presence of oedema, hemorrhages and extensive necrotic areas on the stumps of abnormal limbs supported this view-point. The placentae and amniotic membranes showed normal morphological features and therefore could not be implicated.

Singh and Singh (1973) subjected Wistar rats to amniocentesis using puncture instruments of three different diameters. The punctures were accompanied by the withdrawl of 0.04 ml of the liquor. To remove the effect of uterine contractions a group of fetuses were removed from the

uterus with placenta and amniotic sac intact, and treated while being maintained in normal saline at 37°C. They found that the intensity and the frequency of limb hemorrhages correlated with the size of the puncture and that none of the fetuses treated ex-utero showed any limb hemorrhages. The causal mechanism was postulated to be compression of the fetus by uterine muscular contraction following evacuation of the amniotic fluid. This caused the hemorrhages and subsequent necrosis with varying degrees of limb degeneration.

Love and Vickers (1973) compared the dysmelias induced by vasopressor agents to those induced by amniocentesis. The vasopressin-dysmelia was attributed to plethora by Jost et al. (1964; cited in Love and Vickers, 1973) and to vasospasm (Davies and Robson, 1970). Poswillo and Sopher (1971) suggested that the two dysmelias represented the same phenomenon and used a common term "acroblapsie" for both. Similar types of lesions have been produced by Petter et al. (1971, cited in Love and Vickers, 1973) by reducing the oxygen tension in the fetus, and by Leist and Grauwiler (1974) by clamping the main uterine vessels of pregnant rats. Petter et al. suggested that hypoxia was the common factor in acroblapsie of two different aetiologies.

Light microscopic comparison of the abnormal limbs of fetuses in which either one or the other syndrome had been induced revealed major differences between the dysmelias. Although hemorrhages were common to and considered to be partially responsible for both types

of lesion, it was concluded that the two syndromes, at least in part, result from two distinct processes.

2.3.2 Clinical Aspects of Amniocentesis

Amniocentesis is the insertion of a needle into the amniotic sac. The preferred route of insertion in later pregnancy (from 14 weeks onward) is the transabdominal route. A transvaginal route, which is more difficult and has a higher risk of complications, was described by Fairweather (1973). It was recommended for use only in the early weeks of pregnancy (12-15 weeks) and only in cases where the interruption of pregnancy may be warranted. In a joint statement regarding the indications and the management of amniocentesis (Hamerton et al., 1974) the Canadian Medical Association stated that vaginal amniocentesis or amniocentesis prior to 14 weeks gestation is associated with an unacceptably high complication rate and is not recommended.

At amniocentesis, fluid may be removed for investigation or conversely procedures such as erythrocyte transfusion, catheters to measure fluid pressure, hypertonic saline to induce abortion, or dyes for further investigation may be administered (Fairweather, 1973).

Amniocentesis was first advocated for diagnostic purposes in the practice of obstetrics in 1930 by Mendees et al. (cited in Fairweather, 1973) when they published an account of 21 amniograms. In 1937, Abdurel reported the use of amniocentesis for injecting hypertonic saline to induce therapeutic abortion (cited in Fairweather, 1973). The withdrawl of liquor amnii after puncturing the amniotic sac has been used therapeutically

in hydramnios for more than a century (see Fuchs, 1970). In the past 20 years spectophotometric analysis of liquor removed following amniocentesis has been used routinely in the management of pregnancies complicated by Rhesus or ABO isoimmunization. By identifying the sex chromatin in fetal cells suspended in the liquor, fetuses at risk with sex-linked genetic disorders can be identified (Makowski et al., 1956; Sachs et al., 1956). Many biochemical and genetic tests have been evolved whereby other fetal disorders such as open neural tube defects and inborn errors of metabolism can be identified pre-natally. Indeed, intra-uterine diagnosis and treatment have assumed an increasing importance in the management of metabolic and cytogenetic defects (Poswillo, 1972).

The theoretical possibility of damage to the fetus from amniocentesis was first suggested by Dewhurst in 1956 and experimental evidence to support this view point was given by Trasler et al., also in 1956. It is known and accepted that there is some risk both to the mother and fetus in amniocentesis. The risks vary according to the route employed and to the time of gestation at which amniocentesis is performed. For the mother they include infection, trauma, and hemorrhage. The risks to the fetus include abortion, premature delivery, trauma, sensitization due to maternal bleeding and induced abnormalities (Fairweather, 1973). Nadler (1970) has suggested that unless the risk of a malformation is greater than 1 to 2%, amniocentesis is contraindicated since the risk inherent in amniocentesis itself would be greater than the initial threat.

Unfortunately, few reports of congenital malformations associated with amniocentesis in humans have been published, although considerable concern has been expressed in this regard (Aladjem, 1969). If the lack of such reports can be taken to indicate the safety of the method then Nadler and Gerbie (1971) are justified in stating that when carefully employed, amniocentesis has negligible (< 1%) maternal or fetal morbidity and mortality.

An investigation by Poswillo (1972) in which experimental first-trimester amniocentesis was performed on non-human primates (Macaca irus), failed to produce the congenital defects that have been produced in lower mammalian species. The factor protecting the fetus of M. irus from the amniocentesis syndrome however, may be the ability of the extra-embryonic-coelomic fluid to coagulate within seconds even in the presence of the anti-coagulating properties of Sequestrene thus providing a self-sealing mechanism. Poswillo concluded that if the need should arise for intra-amniotic injection or aspiration midway through the first trimester for either diagnostic or therapeutic purposes, the hazard of induction of congenital malformation may be less than has been predicted on the basis of previous animal experiments.

SECTIONS 1 - 4

METHODS AND MATERIALS

3. METHODS AND MATERIALS

3.1 ANIMALS

Albino Spraque-Dawley rats of the Holtzman strain, weighing 180-220 g, were obtained from Biobreeding Laboratories in Ottawa for use in this study. The animals were kept in wire-mesh cages under controlled environmental conditions (temperature 68-72°F; relative humidity approximately 50%) and maintained on Teklab Mouse and Rat diet (6%) and water, both available ad libitum.

Male rats were placed with virgin females overnight and the following morning vaginal smears were taken and examined for the presence of spermatozoa. The first day of gestation was considered to be the day on which spermatozoa were found in the vaginal smear (Kalter, 1968).

3.2 PRELIMINARY INVESTIGATION

3.2.1 Experimental Design

Experiments were designed to evaluate the usefulness of the intra-amniotic route of administration of test substances in teratological studies.

On days 14, 15 or 16 of gestation pregnant females were anaesthetized with sodium pentobarbital (Nembutal) administered intraperitoneally. Laparotomy was performed and both uterine horns exteriorized using sterile procedures. The number of amniotic sacs and resorption sites if present, were recorded separately for each uterine horn. In all cases the right horn was used as the control side and the fetuses of the left

horn were the treated experimental group. In addition, one group of rats received no treatment at all and served as a further control. The fetuses in the experimental uterine horns were treated as shown in Table I (see also Description of Treatment Procedures below). Each treatment group for each treatment day consisted of three rats (10-25 fetuses). Following treatment, the uterine horns were returned carefully to the peritoneal cavity, the abdominal wall sutured in two layers using sterile, silk Ethicon (4-0) sutures after which the animals were returned to their cages to recover.

Table I. Preliminary investigation. Treatment schedules*

	control/right	experimental/left
I.	no treatment/control	no treatment/control
II.	not punctured	not punctured
III.	not punctured	punctured (26 g needle)
IV.	not punctured	punctured (26 g needle) with injection of 50 µl. sterile water
V.	not punctured	punctured (26 g needle) with injection of 100 µl. sterile water

*each treatment schedule was administered on each of days 14, 15 or 16.

Description of Treatment Procedures.

I. Control group - no treatment at all.

II. Not punctured - anaesthesia, laparotomy, exteriorization of uterine horns etc., but the amniotic membranes were not punctured.

III. Punctured - the amniotic sacs were punctured on the antimesometrial surface with a 26 g 1/2" sterile, disposable Yale hypodermic needle. Care was taken to avoid vascular damage, pressure on the amniotic sac and forceful entry and exit of the needle.

IV. Puncturing of the amniotic sacs and injection of 50 µl of sterile distilled water.

V. Puncturing of the amniotic sacs and injection of 100 µl of sterile distilled water.

On day 20 of gestation the animals were killed by ether administration and the fetuses were recovered. Resorption sites (excluding those present at the time of treatment) and dead fetuses were recorded. All fetuses recovered were measured (crown-rump length), weighed and examined for gross external malformations. Control and experimental fetuses were placed separately in Bouin's solution. One or two fetuses from each uterine horn were eviscerated and placed in absolute alcohol for subsequent study of the skeletal system after staining with alizarin red S. (Dawson, 1926).

3.2.2 Statistical Analysis

A mixed analysis of variance was applied to the following four fetal parameters for all groups, combining groups treated on gestational days 14, 15 or 16: weight (Wt.), placental index (Cruickshank and Miller, 1923), or fetal weight divided by placental weight (Wt./P.wt.), crown-rump length (Browne, 1924), and the CRL/Wt. ratio. The independent factor was the treatment schedule with five levels and the correlated factor

was the uterus with two levels.

Paired t-tests were done on each fetal parameter individually within the five treatment schedules, again combining the data for days 14, 15 and 16 for each treatment.

Fetal mortality was evaluated by using percentage histograms.

The ratio of maternal weight gain during pregnancy per fetus (g/fetus) was evaluated using an analysis of variance.

3.3 CONTROL OF TERATOGENICITY

3.3.1 Experimental Design

Experiments were designed to investigate the possibility of ameliorating or eliminating the teratogenic effects of puncturing the amniotic sac.

On day 16 of gestation pregnant females were anaesthetized and subjected to laparotomy as previously described with sterile hypodermic needles of three different diameters - a 26 g 1/2" disposable Yale needle, a 30 g 1" stainless steel Yale needle and a 34 g 2" stainless steel Hamilton needle. For needle gauges 26 and 34 an attempt was made to seal the puncture hole with a square of surgical Gelfoam soaked in sterile water. The treatment group for each treatment in the schedule included the fetuses of 5 rats (20-35 fetuses). The fetuses of the right uterine horn were the untreated controls, those of the left were the treated experimental group. Following treatment the fetuses were returned to the peritoneal cavity and the abdominal wall sutured as previously described.

Table 2. Control of teratogenicity. Treatment schedules.

	control/right	experimental/left
I.	not punctured	punctured (26 g 1/2" needle)
II.	not punctured	punctured (30 g 1" needle)
III.	not punctured	punctured (34 g 2" needle)
IV.	not punctured	punctured (26 g 1/2" needle) with Gelfoam seal
V.	punctured (26 g 1/2" needle)	punctured (26 g 1/2" needle) with Gelfoam seal
VI.	punctured (34 g 2" needle)	punctured (34 g 2" needle) with Gelfoam seal

On day 20 of gestation the mothers were killed and the fetuses recovered. The positions in the uterine horns of dead fetuses and resorption sites, as well as the positions of abnormal fetuses were recorded on a chart-diagram for each rat (see Fig. 1). Crown-rump length and weight were recorded for viable fetuses. Experimental and control fetuses were fixed in Bouin's solution.

3.3.2 Statistical Analysis

A mixed analysis of variance, as previously described, was applied to the four fetal parameters, Wt., CRL, Wt./P.wt., and CRL/Wt.) for treatment groups I to IV inclusive.

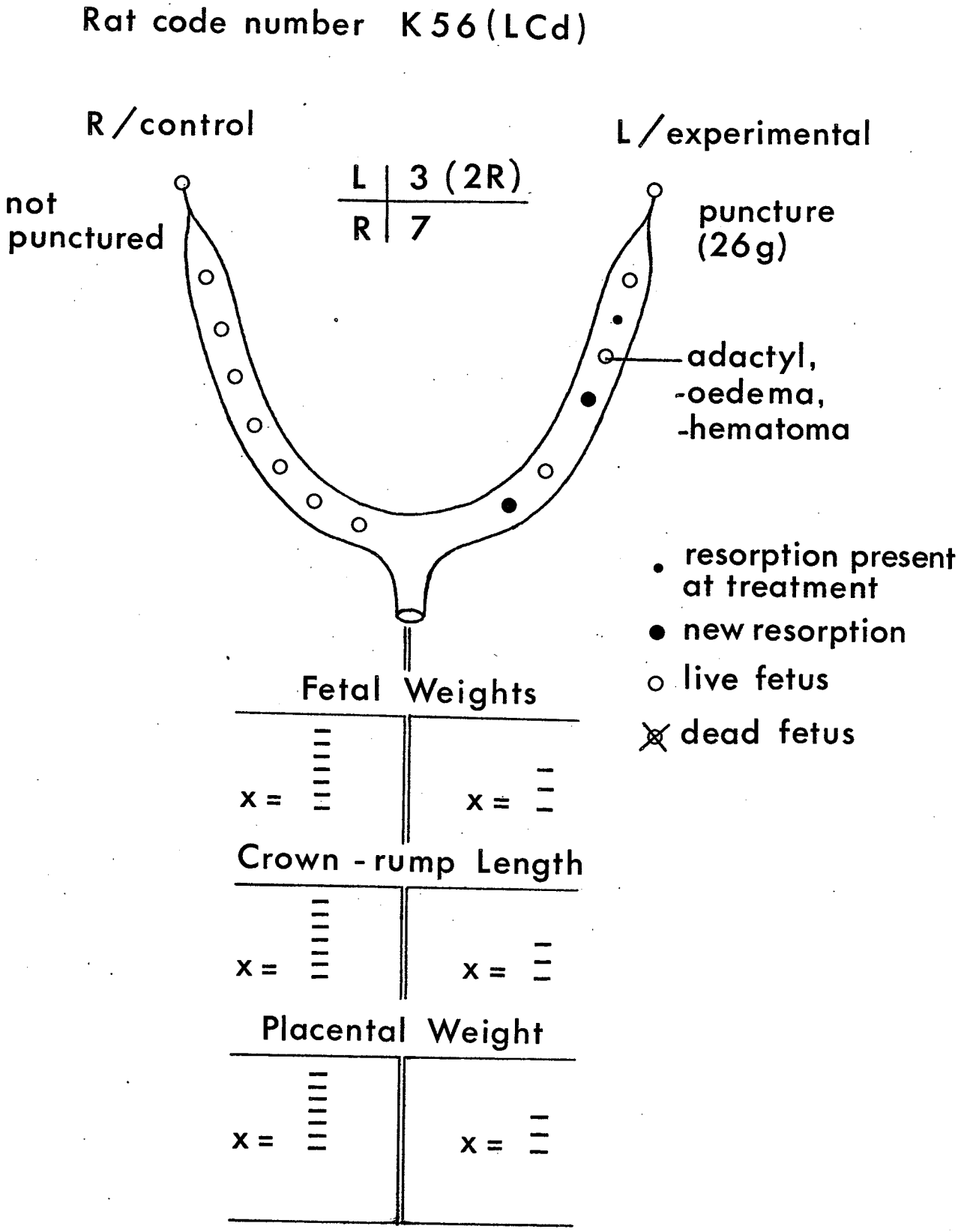


Figure 1. Chart-diagram used to record fetal measurements for individual rats.

A paired t-test was applied to each of the above fetal parameters separately for each of treatment groups I to VI inclusive.

An analysis of variance was used to assess the relationship of maternal weight gain to treatment schedule.

The number of fetal malformations resulting from the treatment schedules was studied in two ways. An analysis of variance was applied to the number of fetal malformations in groups I to IV inclusive. A paired t-test was applied to groups V and VI to study the effect of the attempt to seal the punctures of one set of fetuses, on the number of malformations induced.

3.4 MORPHOGENESIS OF DEVELOPMENTAL DEFECTS FOLLOWING AMNIOTIC SAC PUNCTURE.

On day 16 of gestation pregnant rats were anaesthetized, laparotomy was performed and the uterine horns were exteriorized as previously described. The numbers of amniotic sacs and resorption sites, if present, were recorded at this time. The uterine horn with the most fetuses was treated experimentally. The amniotic membranes of the experimental fetuses were punctured with a 26 g 1/2" sterile, disposable, Yale hypodermic needle. The uterine horns were replaced in the peritoneal cavity and the abdominal wall sutured in two layers as previously described.

The mothers were killed and the fetuses recovered at exact intervals of 15, 30 and 60 minutes, and 12, 24, 36, and 48 hours after treatment. A group of five rats was used for each time interval. The ovaries and

uterine horns were removed, the amniotic sacs were carefully opened and the intact unit was placed immediately in Bouin's solution. The fixed specimens were examined later under a Wild Heerbrugg dissecting microscope for gross external abnormalities which were recorded at that time. Photographs were taken of the lesions detected at each time interval after which the specimens were processed for routine histological studies. Serial sections (7 μ thick) of the limbs and head regions of control fetuses and of the defective limbs and head regions of experimental fetuses were made for each time interval after treatment, stained with hematoxylin and eosin, and examined for microscopic changes.

SECTIONS 1 - 3

RESULTS

4. RESULTS

4.1 PRELIMINARY INVESTIGATION

The mean values for all fetal measurements studied and maternal weight gain are presented in Table 3.

4.1.1 Fetal Measurements (Weight, Crown-rump Length, Placental Index and Length-Weight Ratio

The two-tailed probability for each mean fetal measurement studied (weight, crown-rump length, placental index, length-weight ratio plotted against the individual treatment schedules, is shown in Figure 2. There were no significant differences at or above the 5% level of confidence between the control fetuses recovered from the untreated horns when compared to the experimental fetuses from the treated horns in weight, crown-rump length (CRL), placental index or the ratio between CRL and fetal weight.

The sources of variation as revealed in a mixed analysis of variance, among all parameters in the offspring and the treatment schedules which were administered, are presented in Table 4. The individual treatment schedules produced no significant variations ($p < 0.05$) in weight, CRL, placental index or the CRL/Weight ratio in the experimental fetuses to which they were administered. Between the fetuses of the untreated and treated uterine horns however, there was a highly significant tendency to differ ($p < 0.06$) in weight. There was no significant variation in either the placental index or the CRL/Weight ratio between

Legend for Table 3.

n = number of fetuses

(x) = x dead and/or resorbed fetuses

Wt. = fetal weight

CRL = crown-rump length

Wt./P.wt. = placental index

CRL/Wt. = fetal length-weight ratio

— = no values available

Table 3. Preliminary investigation. Mean fetal and maternal measurements following individual treatments on gestational days 14, 15 or 16.

n	Gestational day	Fetal Measurements Wt. (g).	CRL (cm.)	Wt/P.wt.	CRL/Wt.	Maternal weight gain in g/fetus
I. Control/Control						
23/21		1.8/1.8	2.8/2.8	4.5/4.5	1.6/1.6	8.75
II. Not punctured/Not punctured.						
12/13	14	2.6/2.6	3.2/2.8	5.2/5.2	1.2/1.1	8.6
36/33	15	2.4/2.4	3.0/3.0	4.8/4.8	1.3/1.3	8.78
16/16	16	2.5/2.5	3.1/2.9	4.2/5.0	1.2/1.2	7.5
III. Not punctured/Punctured						
12/17(13)	14	1.95/1.6	3.0/2.7	3.9/4.0	1.5/1.7	9.47
7(3)/11(7)	15	2.0/1.8	3.0/2.8	4.0/4.5	1.5/1.6	7.1
9(2)/17(5)	16	1.8/1.9	2.8/2.7	3.6/3.8	1.6/1.4	7.6
IV. Not punctured/Punctured with 50 µl. injection of sterile water						
11(1)/12(12)	14	1.7/-	2.7/-	5.7/-	1.6/-	6.25
5/9(8)	15	-----	-----	------	------	-----
21/22(5)	16	1.8/1.7	2.8/2.7	4.5/4.3	1.6/1.6	6.85
V. Not punctured/Punctured with 100 µl. injection of sterile water.						
11/13(12)	14	2.3/-	3.0/-	5.8/-	1.3/-	5.9
3/11(11)	15	1.7/-	2.7/-	4.3/-	1.6/-	6.4
4/9(2)	16	2.3/1.7	3.0/3.0	5.8/4.3	1.3/1.8	-----

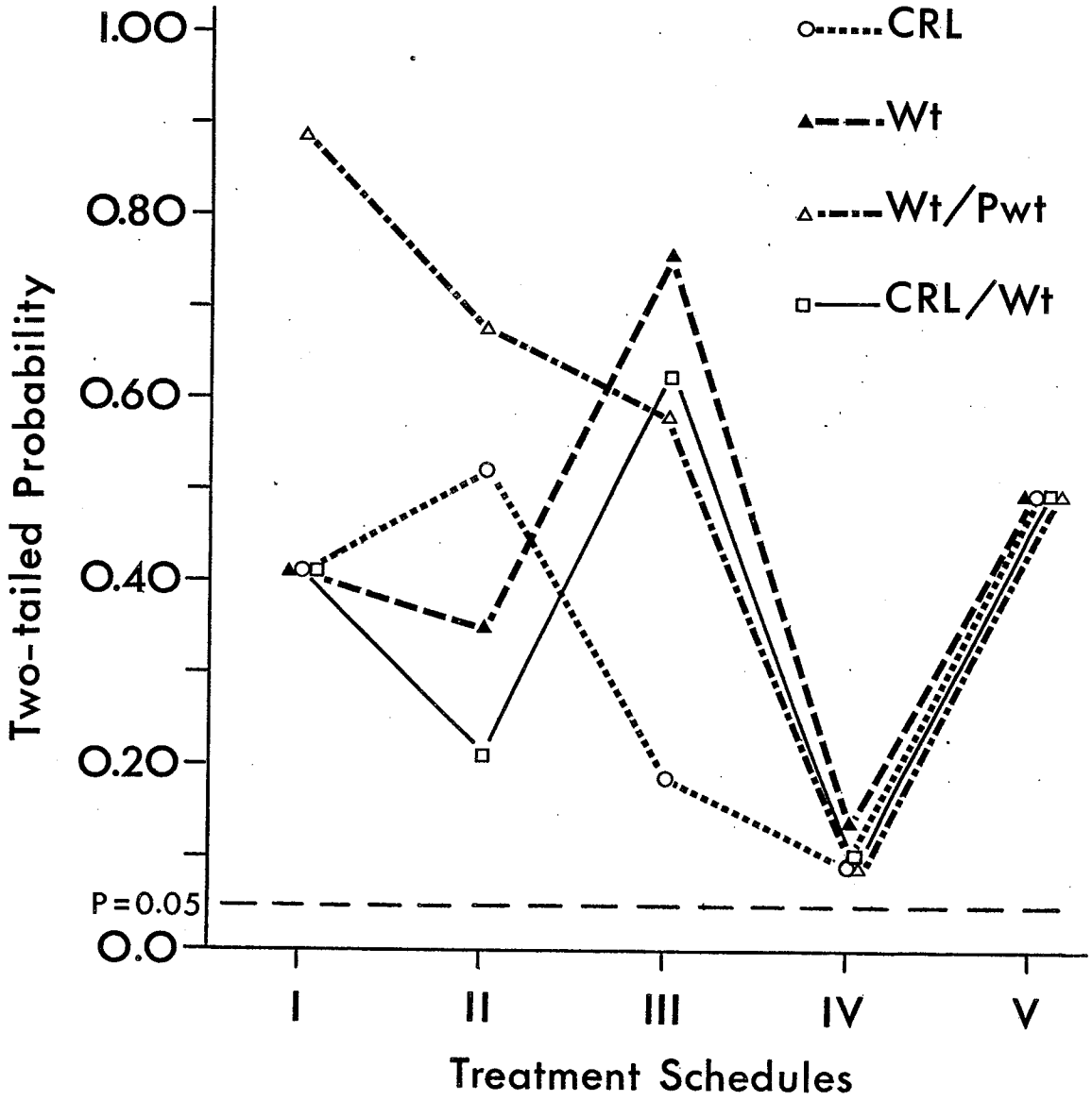


Figure 2. Preliminary investigation. Two-tailed probability for each fetal measurement following the administration of individual treatments.

Table 4. Preliminary investigation. The sources of variation among treatment schedules and fetal parameters as indicated in a mixed analysis of variance.

Source of variation	Level of significance	Required F value
1. Fetal weight		
group F = 1.257	not significant	F > 3.01 @ 0.05
uterus F = 4.106	6.33%*	F > 4.49 @ 0.05
2. Crown-rump length		
group F = 1.799	not significant	F > 3.01 @ 0.05
uterus F = 4.806	5%	F > 4.49 @ 0.05
3. Placental index		
group F = 1.677	not significant	F > 3.41 @ 0.05
uterus F = 0.033	not significant	F > 4.67 @ 0.05
4. CRL/weight ratio		
group F = 1.103	not significant	F > 3.41 @ 0.05
uterus F = 0.464	not significant	F > 4.67 @ 0.05

*achieved by inverse interpolation

treated and untreated fetuses.

The relationships between fetal mortality, individual treatment schedules and gestational age at the time of treatment are presented in Figures 3 to 7. For all three treatment days and all treatment schedules not involving puncture of the fetal membranes (groups I and II) there were no fetal deaths. When the fetal mortality rates for all treatment schedules were combined for days 14, 15, and 16 of treatment, there was a reduction in fetal mortality with increasing gestational age at the time of treatment (Figure 3).

Mortality rates were also found to be treatment-specific (see Figure 4). The overall mortality rate for each treatment schedule, obtained by combining the three gestational days on which each schedule was administered, was found to be specific to the individual treatment schedule. Mortality rates increased from 0% in groups I (untreated) and II (unpunctured) to 74.1% in group V (punctured, with 100 µl injection of sterile water). This clearly reflects a gradient of severity among the treatment schedules which increases from group I through group V. This increasing gradient of treatment severity was also found to exist for treatment days 14 and 15 considered separately, but not for day 16 (see Figures 5, 6 & 7). The low, treatment-independent mortality rate for day 16 again showed that fetal age was an important determinant of fetal mortality.

4.1.2 Maternal Weight Gain

An analysis of variance revealed that there were no significant differences between the maternal weight gains (relative to the number of fetuses in the litter) of the five treatment schedules ($F = 1.6126$,

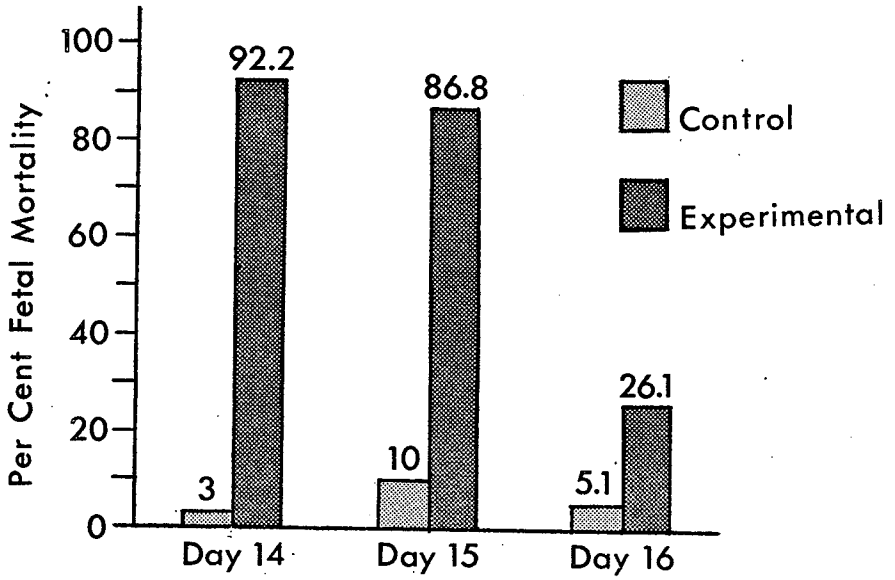


Figure 3. Preliminary investigation. Overall fetal mortality on individual treatment days.

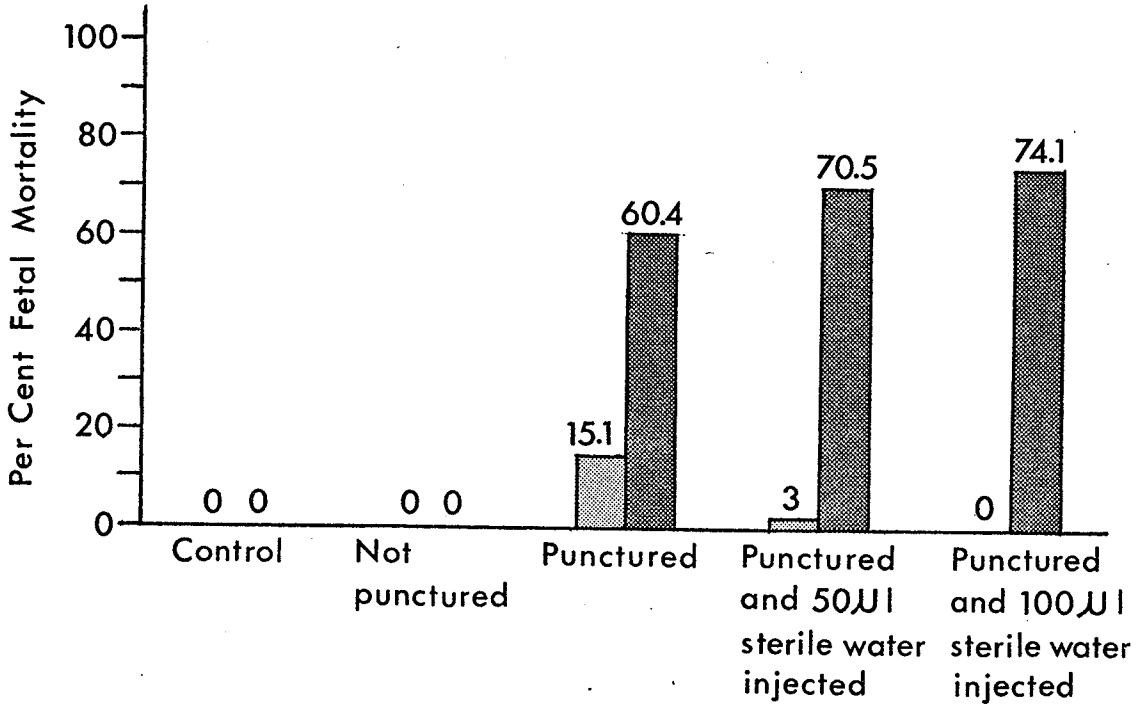


Figure 4. Preliminary investigation. Overall fetal mortality following the administration of individual treatments.

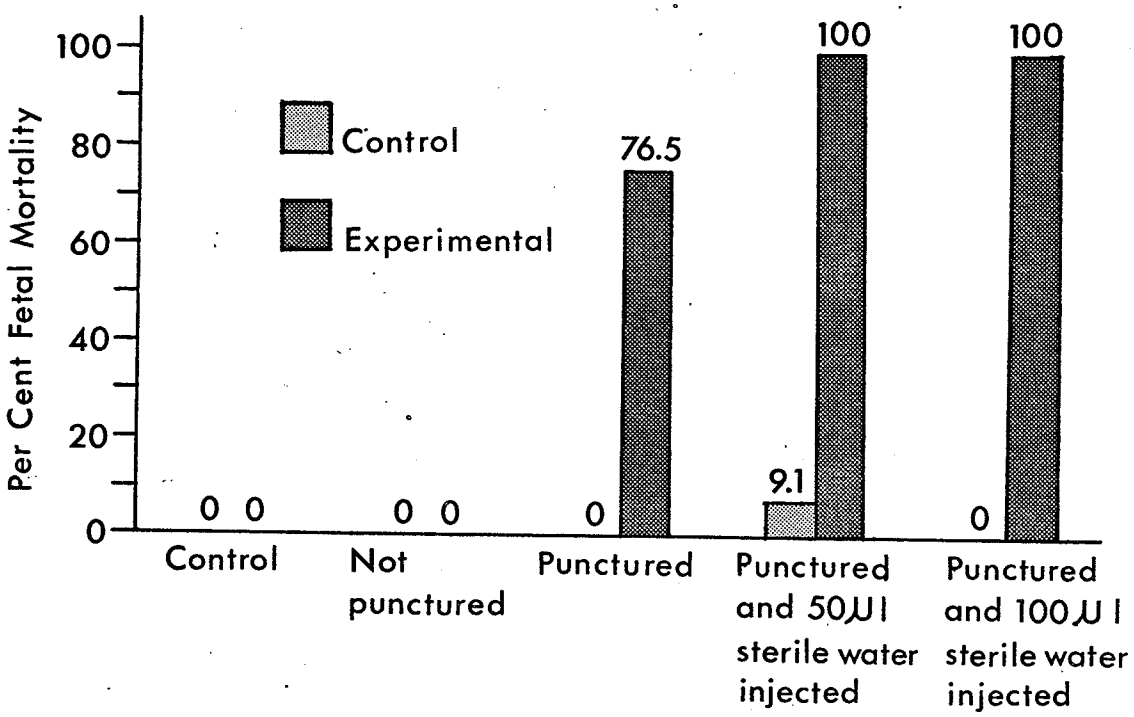


Figure 5. Fetal mortality on gestational day 14 following the administration of individual treatments.

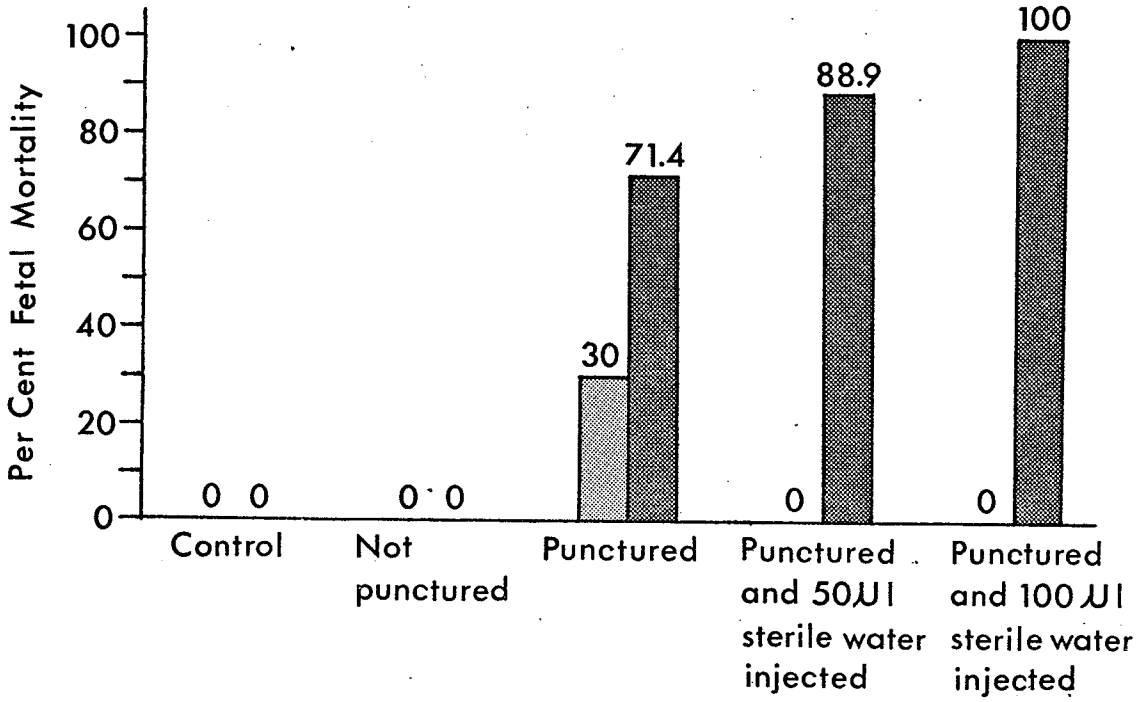


Figure 6. Fetal mortality on gestational day 15 following the administration of individual treatments.

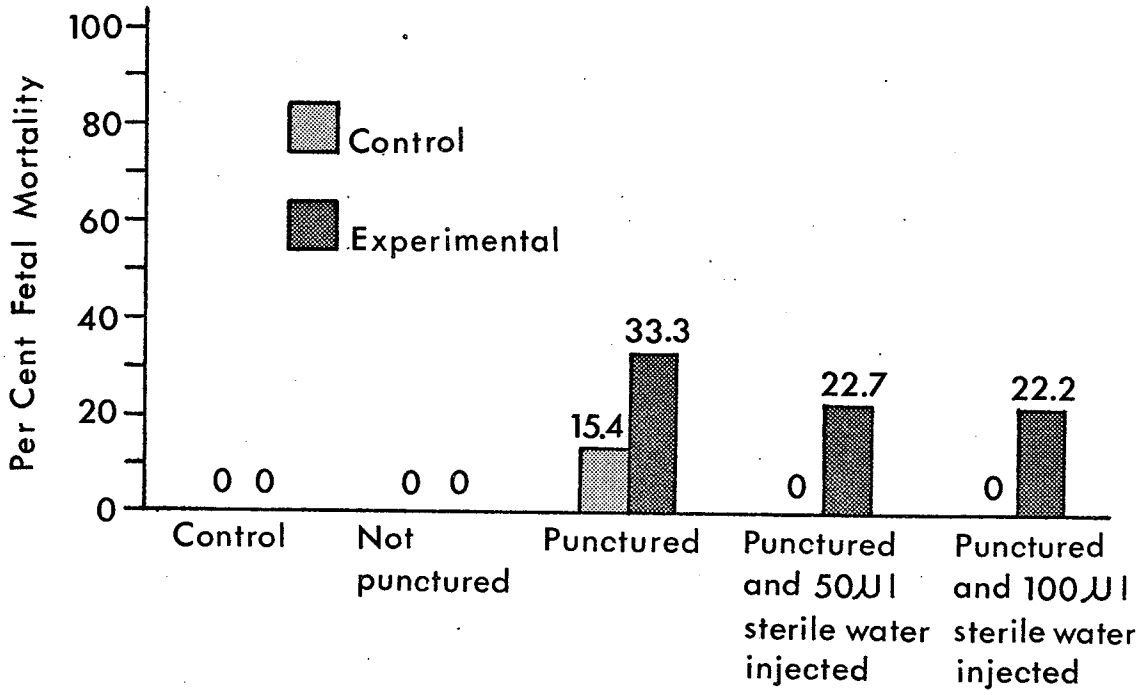


Figure 7. Fetal mortality on gestational day 16 following the administration of individual treatments.

Legend for Table 5.

n	=	number of fetuses
(x)	=	x dead and/or resorbed fetuses
Wt.	=	fetal weight
CRL	=	crown-rump length
Wt./P.wt.	=	placental index
CRL/Wt.	=	fetal length-weight ratio

where the required F> 2.96 @ 0.05, df. 4,17).

4.2 CONTROL OF TERATOGENICITY

The mean values for all fetal measurements studied and for maternal weight gain are presented in Table 5.

4.2.1 Fetal Measurements (Weight, Crown-rump Length, Placental Index and Length-weight Ratio).

The two-tailed probability for each mean fetal measurement studied (weight, crown-rump length, placental index, length-weight ratio) plotted against the individual treatment schedules is shown in Figure 8. Variations between the treated and untreated offspring in weight and crown-rump length followed no consistent pattern and appeared to be group-dependent. In treatment groups II ($p < 0.01$), IV ($p < 0.03$) and VI ($p < 0.04$), the differences in the weights of the treated offspring were highly significant compared to the untreated controls. Groups I, III and V however showed no such variations. In treatment group IV, the difference in CRL was highly significant ($p < 0.001$), but in all other treatment groups there were no significant differences. The placental indices for treated and untreated fetuses showed no significant differences. The variations in the length-weight ratios were highly significant in treatmentgroups I ($p < 0.01$) and VI ($p < 0.02$) between treated and untreated fetuses. In groups II ($p < 0.12$), III ($p < 0.09$) and IV ($p < 0.15$), there were strong tendencies (which were not significant) to differ in this ratio. More than any other parameter studied, the length-weight ratios of treated and untreated offspring showed a

Table 5. Control of Teratogenicity. Mean fetal and maternal measurements following individual treatments on gestational day 16.

	Fetal Parameters					
n.	Wt. (g)	CRL (cm)	Wt./Pwt.	CRL/Wt.	malformed/ normal treated	Maternal weight gain (g/fetus)
1. Not puncture/Punctured (26 g needle)						
28/25(8)	2.2/2.2	3.1/2.9	4.5/4.2	1.4/1.3	$^{8}/_{27}$	10.7
2. Not punctured/Punctured (30 g needle)						
20/19(3)	2.4/2.3	3.1/3.1	4.7/4.9	1.3/1.4	$^{6}/_{15}$	17.44
3. Not punctured/Punctured (34 g needle)						
17/23(3)	2.4/2.4	3.1/3.0	4.8/4.7	1.3/1.3	$^{1}/_{20}$	35.0
4. Not punctured/Punctured (26 g) and seal						
28/27(4)	2.5/2.4	3.2/2.9	5.1/4.8	1.3/1.2	$^{15}/_{23}$	11.0
5. Punctured (26 g)/Punctured 26g) and seal						
20(1)/17(3)	2.3/2.4	3.0/3.0	4.3/4.3	1.3/1.3	$^{9}/_{20}$- $^{3}/_{14}$	14.0
6. Punctured (34 g)/Punctured (34 g) and seal						
23(1)/20	2.5/2.3	3.1/3.0	4.4/4.0	1.2/1.3	$^{2}/_{22}$- $^{5}/_{20}$	11.8

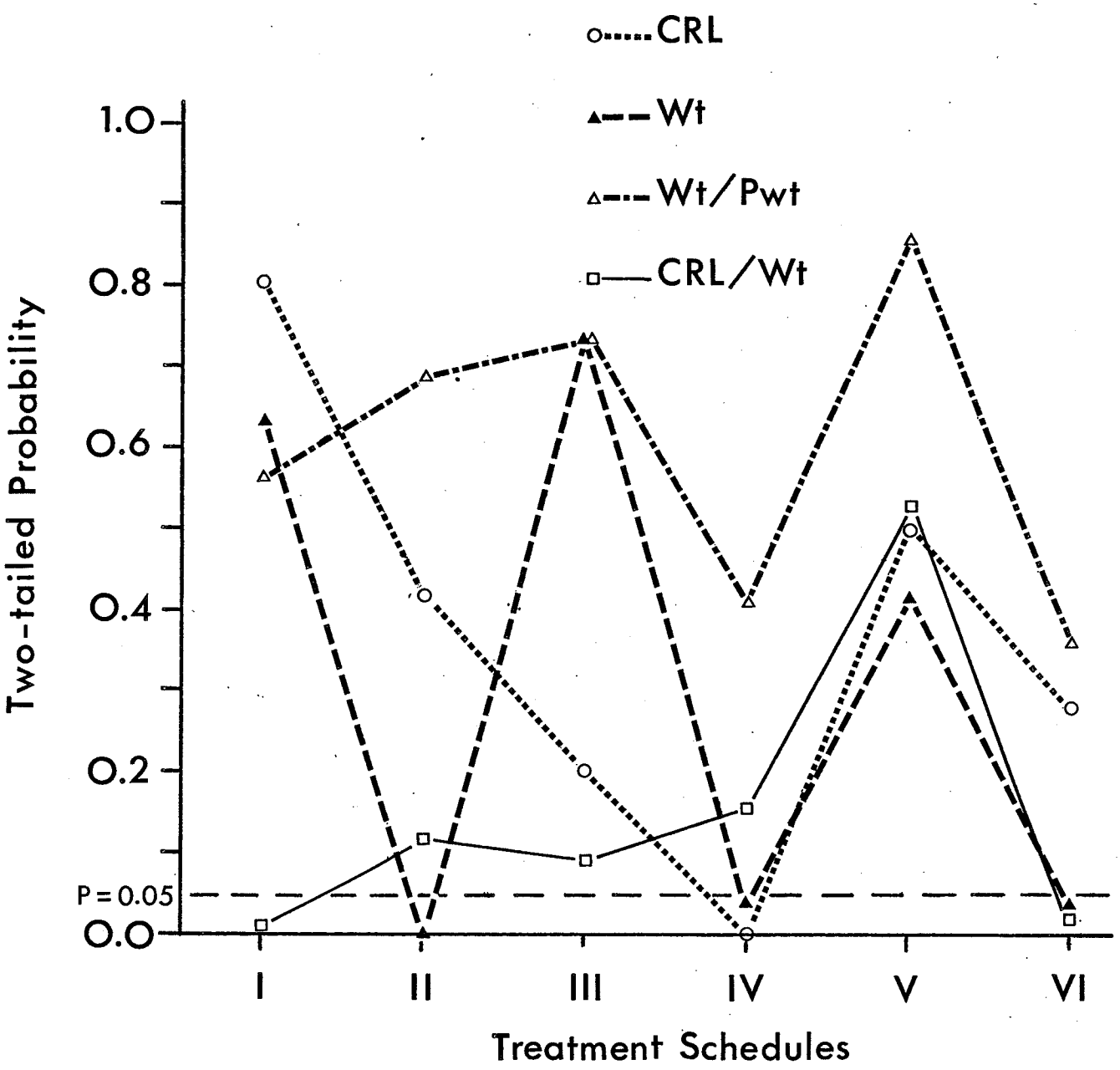


Figure 8. Control of teratogenicity. Two-tailed probability for each fetal parameter measured following the administration of individual treatments.

consistent tendency to differ. All other measures appeared to be group-dependent.

For groups V ($p = 0.155$) and VI ($p = 0.074$) in which the effect of puncture of the amniotic sac was compared to the effect of puncture plus sealing of the puncture sites, the number of abnormal offspring recovered did not differ significantly, nor were there any significant differences in the numbers of malformed offspring between treatment groups I through IV.

The sources of variation as revealed in a mixed analysis of variance, among all measurements studied in the offspring and the individual treatment schedules which were administered, are presented in Table 6. Between treatment groups there were no significant differences in weight, CRL, placental index or length-weight ratio. Between treated and untreated fetuses, the differences in weight ($p < 0.05$), CRL ($p < 0.001$) and in the length-weight ratio ($p < 0.05$) were highly significant, but there was no significant variation in the placental indices. Although there were highly significant differences between the length-weight ratios of untreated and treated fetuses, the variations did not follow the same pattern in the different treatment groups.

The fetal mortality rates associated with individual treatment schedules are presented in Figure 9. There were no fetal deaths or malformed offspring among the control or the unpunctured treated fetuses. There was a corresponding decrease in the incidence of fetal deaths as the diameter of the needle used to puncture the amniotic sac was

Table 6. Control of teratogenicity. Sources of variation among treatment schedules and fetal measurements as demonstrated by a mixed analysis of variance.

Source of variation	Level of significance	Required F value
1. Fetal weight		
group F = 1.438	not significant	F > 3.59 @ 0.05
uterus F = 7.939	5%	F > 9.65 @ 0.01
2. Crown-rump length		
group F = 1.165	not significant	F > 3.59 @ 0.05
uterus F = 34.910	1%	F > 19.7 @ 0.001
3. Placental index		
group F = 1.668	not significant	F > 3.59 @ 0.05
uterus F = 0.457	not significant	F > 4.84 @ 0.05
4. CRL/weight ratio		
group F = 2.053	not significant	F > 3.59 @ 0.05
uterus F = 7.181	5%	F > 9.65 @ 0.01
group uterus F = 8.049	5%	F > 9.65 @ 0.01

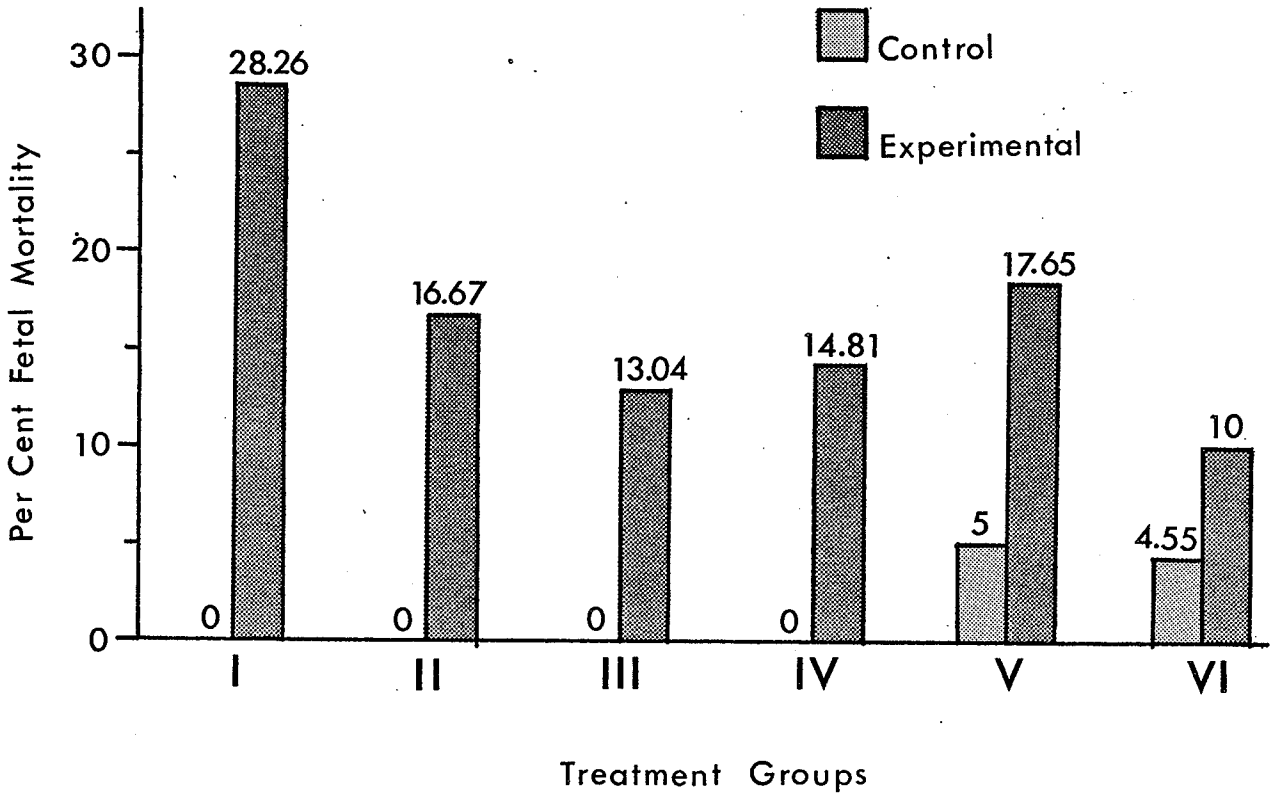


Figure 9. Fetal mortality following the administration of individual treatment s on day 16 of gestation.

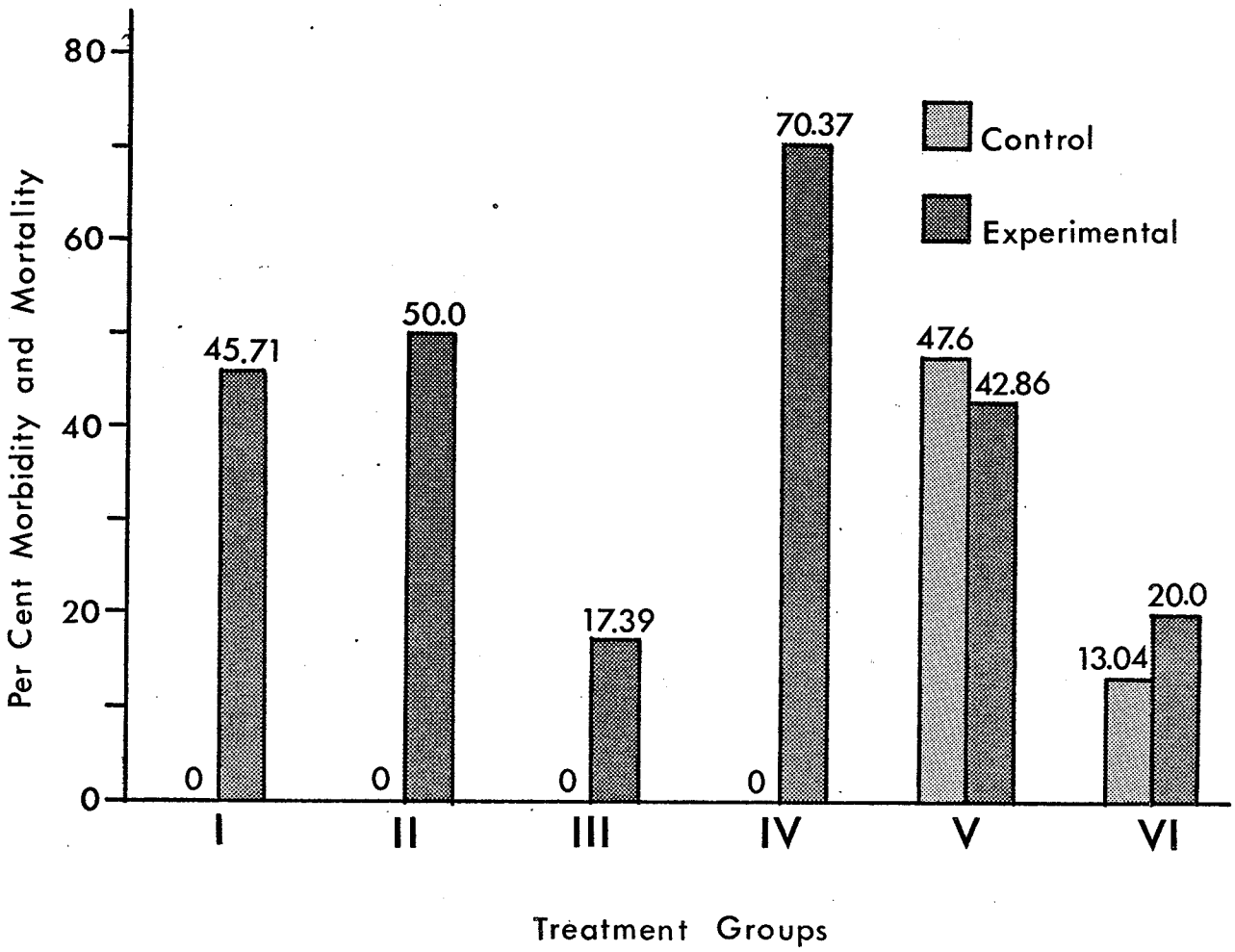


Figure 10. Combined fetal mortality and morbidity following the administration of individual treatments on day 16 of gestation.

reduced (groups I, II & III).

Groups I and IV were both punctured with a 26 g needle however, in group IV an attempt was made to seal the puncture sites with Gelfoam. The incidence of fetal mortality in group IV (14.81%) approximated that of groups II (16.67%) and III (13.04%) which were punctured with needles of smaller calibre. The fetal mortality rate following puncture with a 26 g needle on day 16 of gestation (28.26%) approximated both the 33.3% rate found in the Preliminary Investigation for the same treatment schedule, and the overall 26.1% rate for the combined treatments administered on day 16, also in the Preliminary Investigation.

Groups V and VI both showed a higher incidence of fetal deaths in the uterine horns in which an attempt was made to seal the sites of puncture than in those which were only punctured. However, there was a slightly lower rate for group VI (punctured with a 34 g needle), than for group V (punctured with a 26 g needle). The difference however was not as great as the difference between groups I and III.

When the mortality and morbidity rates were combined, no treatment-specific effect could be seen for the different treatment schedules (see Figure 10).

4.2.2 Maternal Weight Gain

An analysis of variance revealed no significant differences in maternal weight gains (measured in grams per fetus) during pregnancy either between or within the treatment groups ($F = 0.9019$, where the required $F > 2.71$ @ 0.05, df. 5,20).

4.3 MORPHOGENESIS OF DEVELOPMENTAL DEFECTS FOLLOWING AMNIOTIC SAC PUNCTURE

4.3.1 Gross Malformations

4.3.1.1 Early changes

The morphological changes most commonly seen in fetuses recovered 15, 30 and 60 minutes after treatment were vascular damage, postural moulding and a general tissue fragility (Figures 11 to 16). The latter was taken to indicate tissue dessication. The frequency with which the hemorrhages occurred and their severity increased with the time interval after treatment. Subcutaneous hemorrhages were found on the feet as well as on various parts of the limbs which were likely to be subjected to pressure by the contracting uterus (Figures 11b, 13 and 15). Many instances of generalized subcutaneous hemorrhage (Figure 13), subcutaneous hemorrhage related to the spinal column (Figure 11a) and hemorrhages of varying severity on the head (Figures 11b, 12 and 16) which were either subcutaneous or intracranial were observed.

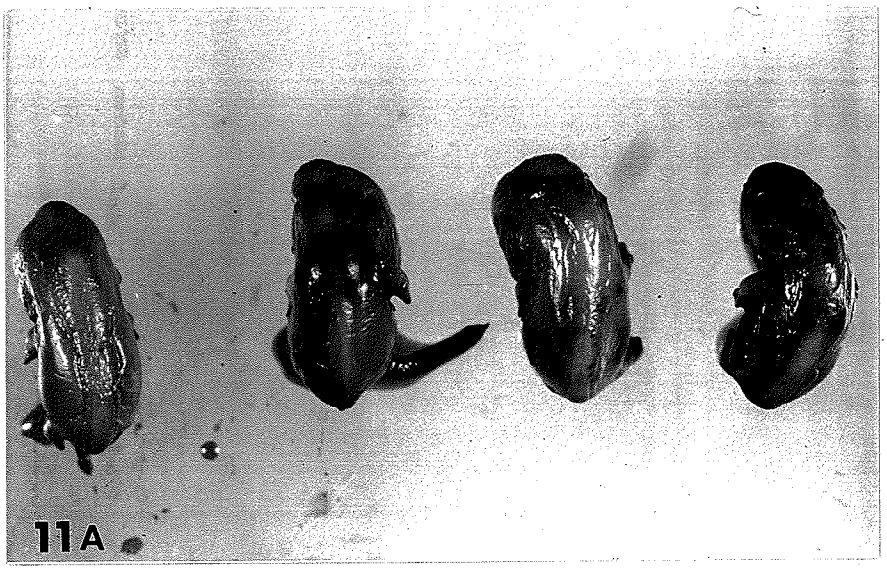
4.3.1.2 Later changes

Fetuses recovered at 12, 24, 36 and 48 hours following amniotic sac puncture showed clearly defined gross malformations mainly of the head and limbs. Where malformations existed they were generally multiple. The severity of the deformities tended to increase with increasing time after puncture and the lesions became predominantly necrotic and regressive in nature.

Figure 11. Extreme left: Control fetus.
Right: Fetuses showing compressive changes 15 minutes after amniocentesis.

(a) Cranioschisis, generalized subcutaneous hemorrhages and malrotation of the spine.

(b) Severe compression, cranial hemorrhage and pooling of blood in the umbilical cord.


11A

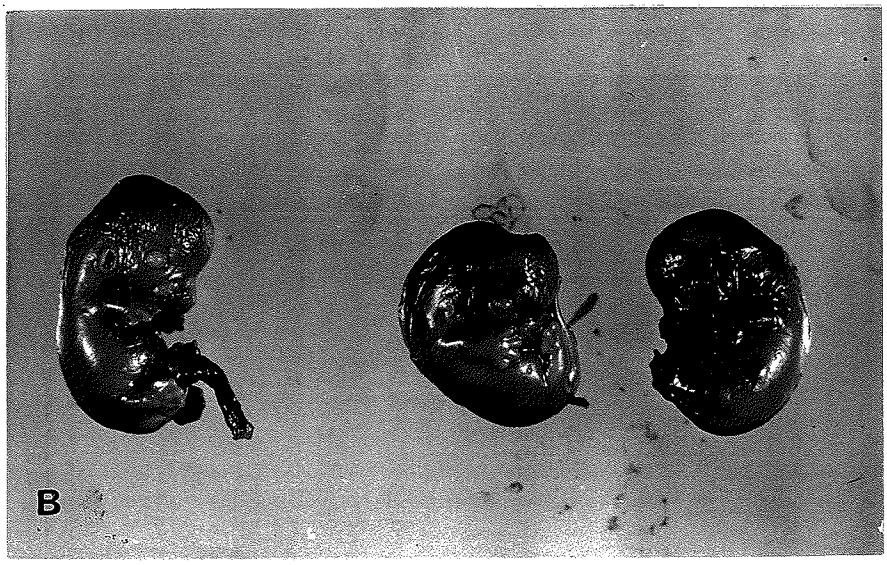

B

Figure 12. Cranioschisis, subcutaneous hemorrhage and dessication observed 15 minutes after amniocentesis.
Control fetus on extreme left.

Figure 13. Wide spectrum of malformations found in the fetuses of one litter 30 minutes after amniocentesis; cranioschisis, generalized and localized subcutaneous hemorrhage and dessication.
Control fetus on the extreme left.

12

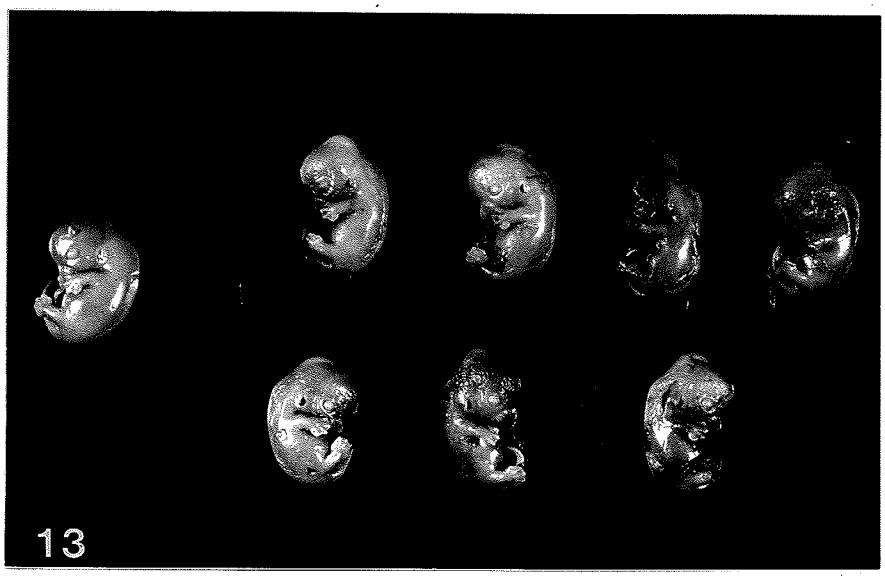


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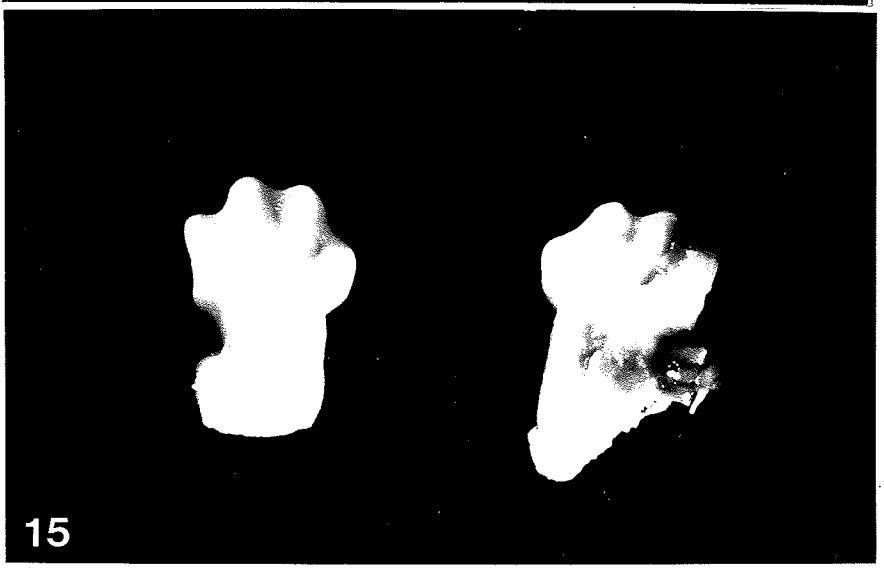
Figure 14. Oedematous fetus with hind limb amputation recovered 60 minutes after amniocentesis.

Figure 15. Left: Control limb.
Right: Subcutaneous hemorrhage and tissue dessication in a limb 15 minutes after amniocentesis.

Figure 16. Control on extreme left.
Right: Presence of hemorrhage in the heads of fetuses recovered 15 minutes after amniotic sac puncture.

14



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16

Postural moulding, suggestive of compression, was frequently observed in treated fetuses. A generalized oedematous appearance, tissue fragility and localized areas of tissue constriction or banding often accompanied the postural abnormalities (Figure 17). Postural abnormalities included malrotations of the limbs (Figure 18), scoliosis or twisting of the spinal column (Figure 11a) and accentuated flexion of the spinal column (Figure 21). This severe flexion resulted in a distortion of the normal relationship between body parts (Figure 17) and was associated with abnormalities of the head and umbilical cord.

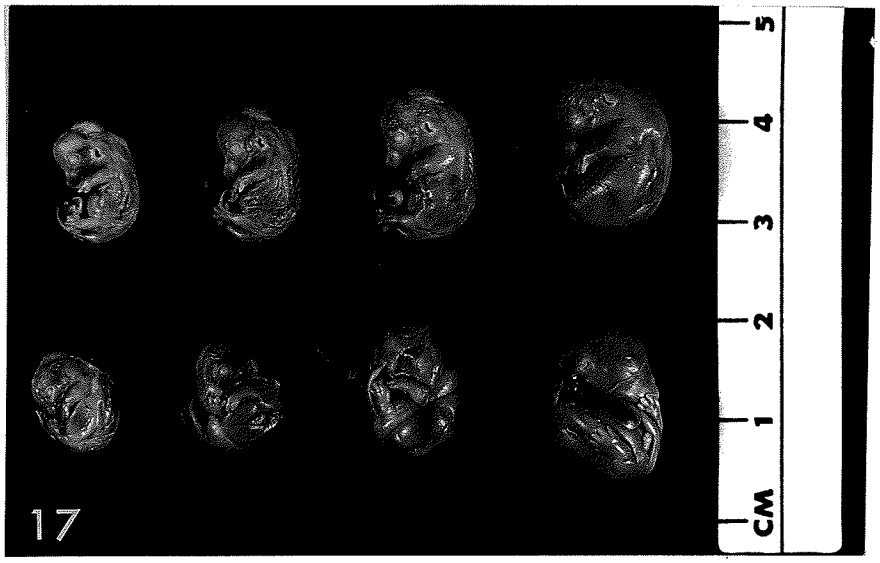
Hemorrhagic lesions were found in three forms; a) localized or generalized subcutaneous hemorrhages, b) large blebs filled with clear fluid (Figure 19b), and c) large blebs filled with blood (Figure 21). These lesions were most frequently observed on the more distal segments of the fetal limbs but were also found on the head and in relation to the spinal column (Figure 22).

Abnormalities detected in the limbs included meromelia, hemorrhagic lesions and blebs, and malrotations (Figures 18, 19 and 20). One case of an amputated limb was seen and the amputated portion was recovered (Figure 14).

In the head region the malformations observed included an abnormal cranial protrusion which was interpreted as being cranioschisis, micrognathia microstomia, and both subcutaneous and intra-cranial hemorrhages (Figures 12, 16 and 18).

Figure 17. Above: Control fetuses.
Below: Compression, oedema and tissue constriction or banding observed in fetuses recovered from 12 to 48 hours after puncture of the amniotic sac.

Figure 18. Malrotation of the limbs, micrognathia, and tissue dessication seen at 12 and 24 hours after amniocentesis.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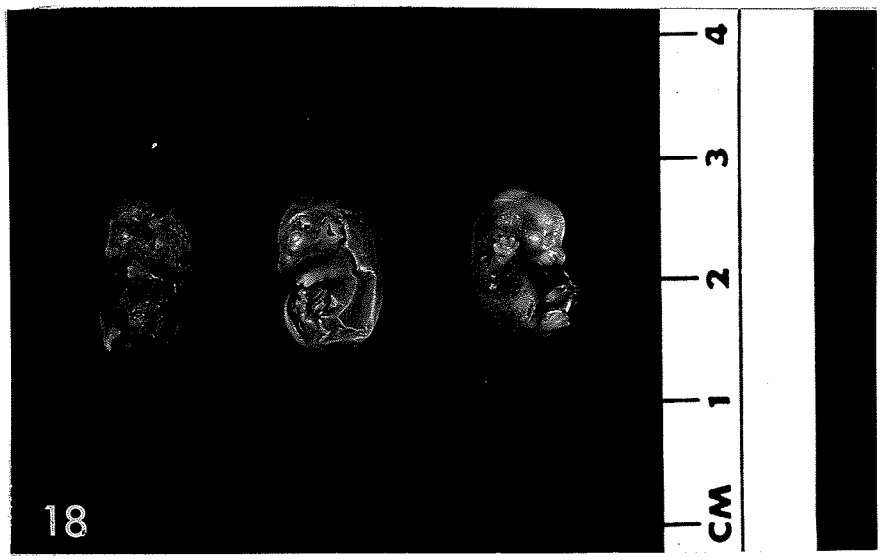
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CM 1 2 3 4

Figure 19. Limb abnormalities observed 36 hours after amniocentesis. Control on left.

(a) Digital reduction and necrosis.

(b) Fluid-filled bleb on dorsum of foot.


19A

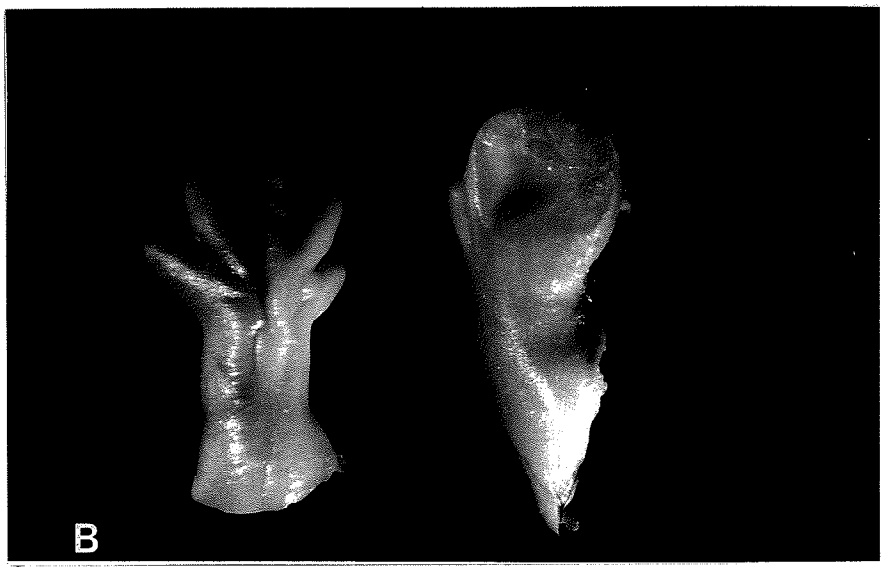
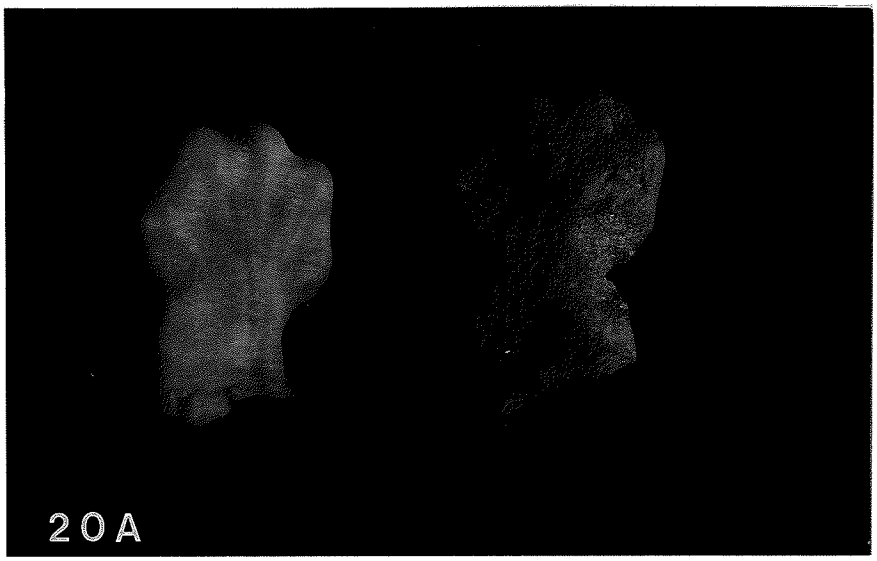

B

Figure 20.

(a) Oedema and dessication of the foot as observed 12 hours after amniocentesis (right). Control on left.

(b) Hemorrhage, oedema and reduction of the digits observed 48 hours after amniocentesis (right). Control on left.


20A

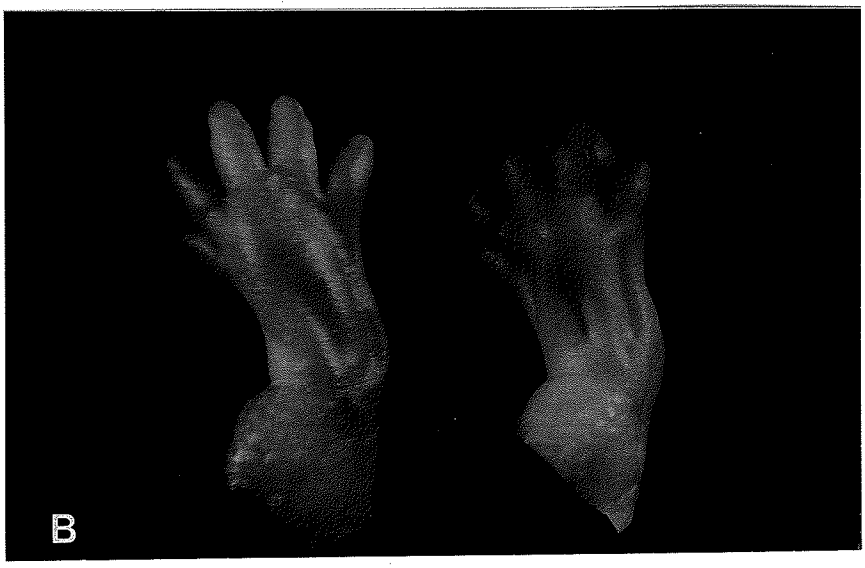
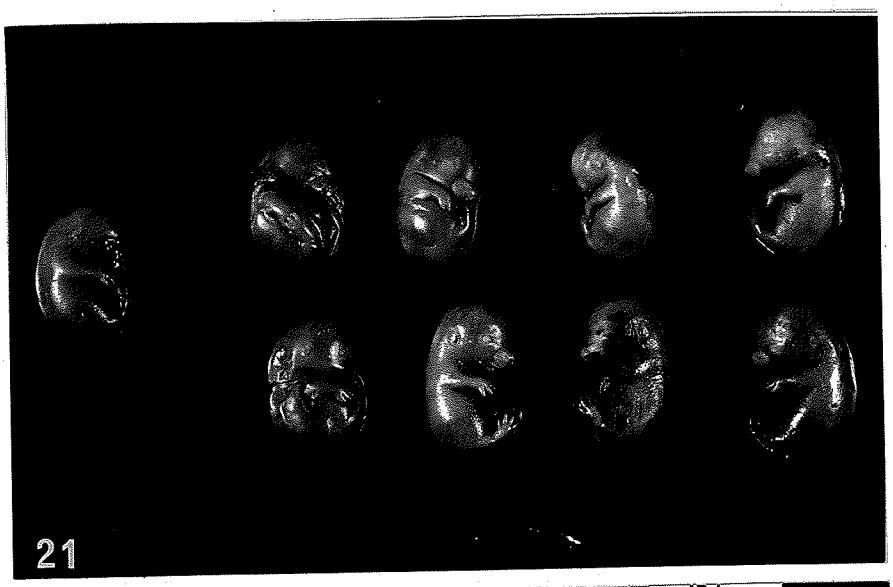

B

Figure 21. Control fetus on extreme left.
Right: Malformations observed in one litter 48 hours following puncture of the amniotic sac; compression, banding, subcutaneous hemorrhage, hemorrhagic foot blebs, dessication, as well as apparently normal experimental fetuses.

Figure 22. Above: Control fetuses.
Below: Spinal bleb observed at 12 hours (right) and spinal hemorrhage found at 24 hours after amniocentesis.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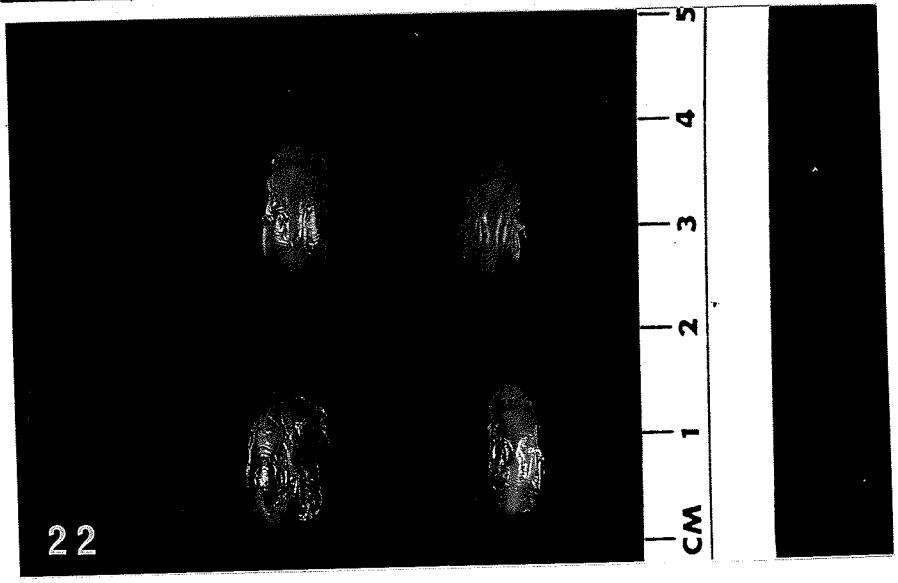
22
CM
1
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Deformities were also found in the abdominal region specifically related to the umbilical cord. Many cases of umbilical compression was seen in the form of a flattened appearance and extensive pooling of blood (Figure IIb). In comparison to untreated fetuses of the same litter the normal herniation of the gut into the umbilical cord was frequently reduced or absent in offspring subjected to amniotic sac puncture.

4.3.2 Light Microscopic Changes

4.3.2.1 Early changes

The earliest morphological changes observed in the limbs and heads of treated fetuses were related to the blood vessels. A pattern of vascular engorgement and disruption, hemorrhage, interstitial oedema, and foci of cells with evidence of cloudy swelling were observed in the fetal tissues recovered within the first hour following amniotic sac puncture (Figures 23 to 25). Massive vascular engorgement of the marginal and deeper blood vessels was the most pronounced change in both the limbs and heads 15 and 30 minutes after puncture of the amniotic sac (Figure 23). Areas of discontinuity in the engorged vessels were found, accompanied by hemorrhage into the adjacent tissues. After one hour, individual cells in the tissue near hemorrhagic sites were markedly separated indicating interstitial oedema, and appeared to be larger and less eosinophilic than both the cells of tissues more

distant from the hemorrhage and the cells of normal tissues as seen in the control fetuses. No nuclear changes indicative of cell death were observed but the presence of cloudy swelling suggested the beginning of cell death (Figure 25d). In some areas interstitial oedema was so marked as to produce areas of cavitation in which only a few free-floating cells were seen. Cavities were also found to exist between tissue layers which were normally continuous. This separation of tissue layers was most pronounced in the head regions of fetuses recovered at the early intervals and again reflected an excessive accumulation of fluid (Figure 25a). These cavities were lined with torn and fragmented blood vessels and tissues.

Necrotic changes were also observed in the interdigital areas of the limbs of untreated fetuses (Figure 26). This physiological necrosis reflects a sculpturing process which is embryologically normal. It differed microscopically from the lesions present in the limbs of treated fetusus in that a) there was no vascular engorgement, discontinuity or hemorrhage in the surrounding tissues, b) many mitotic figures were present indicating a formative or regenerative process, c) there was no interstitial oedema, separation of tissue layers or evidence of cell death in the adjacent tissues, and e) it was found only in the interdigital areas immediately under the epidermis.

4.3.2.2 Later changes

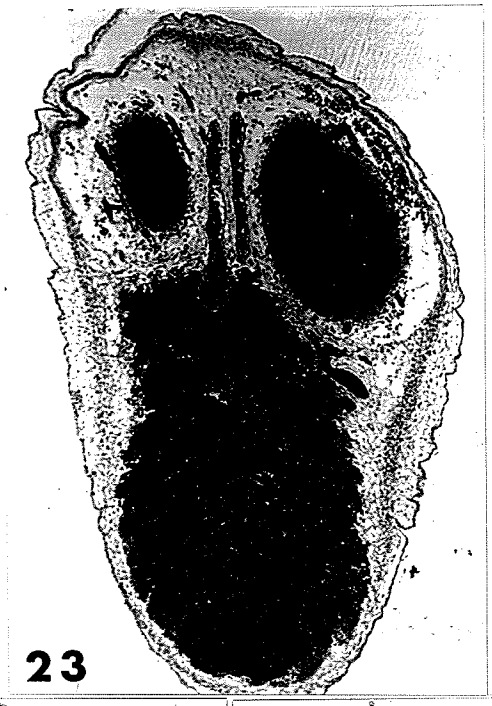
Twelve hours after the amniotic sac was punctured, the process of vascular engorgement and disruption, hemorrhage, and interstitial oedema

Figure 23. Massive intra-cranial hematoma in association with vascular engorgement and separation of connective tissue layers observed 15 minutes after amniocentesis. (x 24)

Figure 24. Vascular lesions in a limb 15 minutes after amniocentesis.

(a) Hematoma associated with vascular engorgement. (x 47)

(b) Engorgement and dilation of marginal blood vessels. (x 38)



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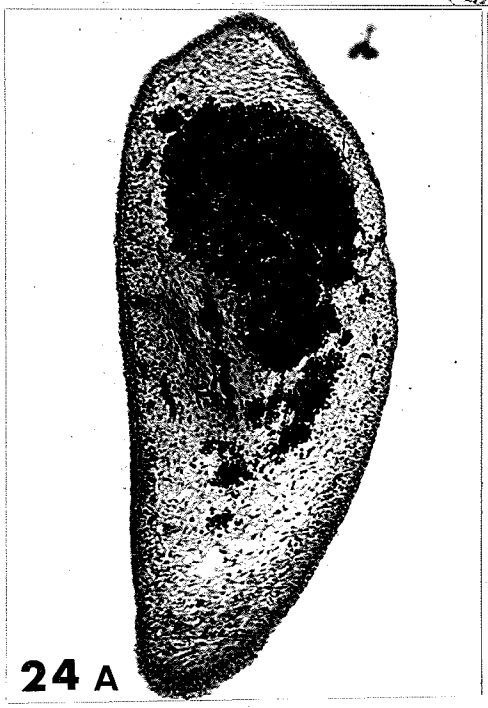
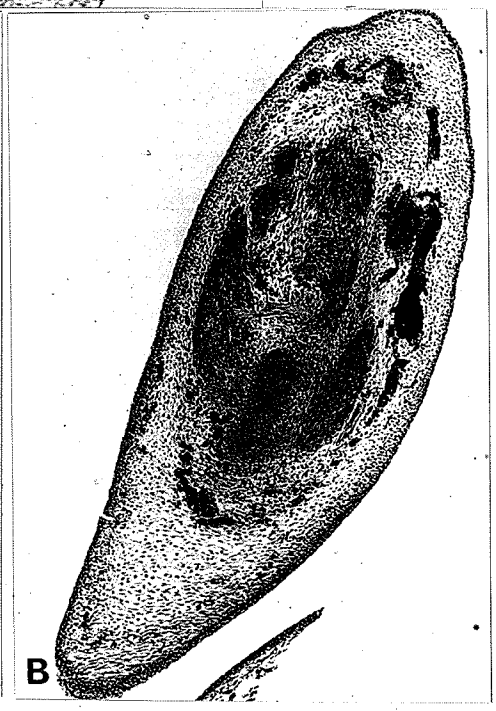


24 A



B

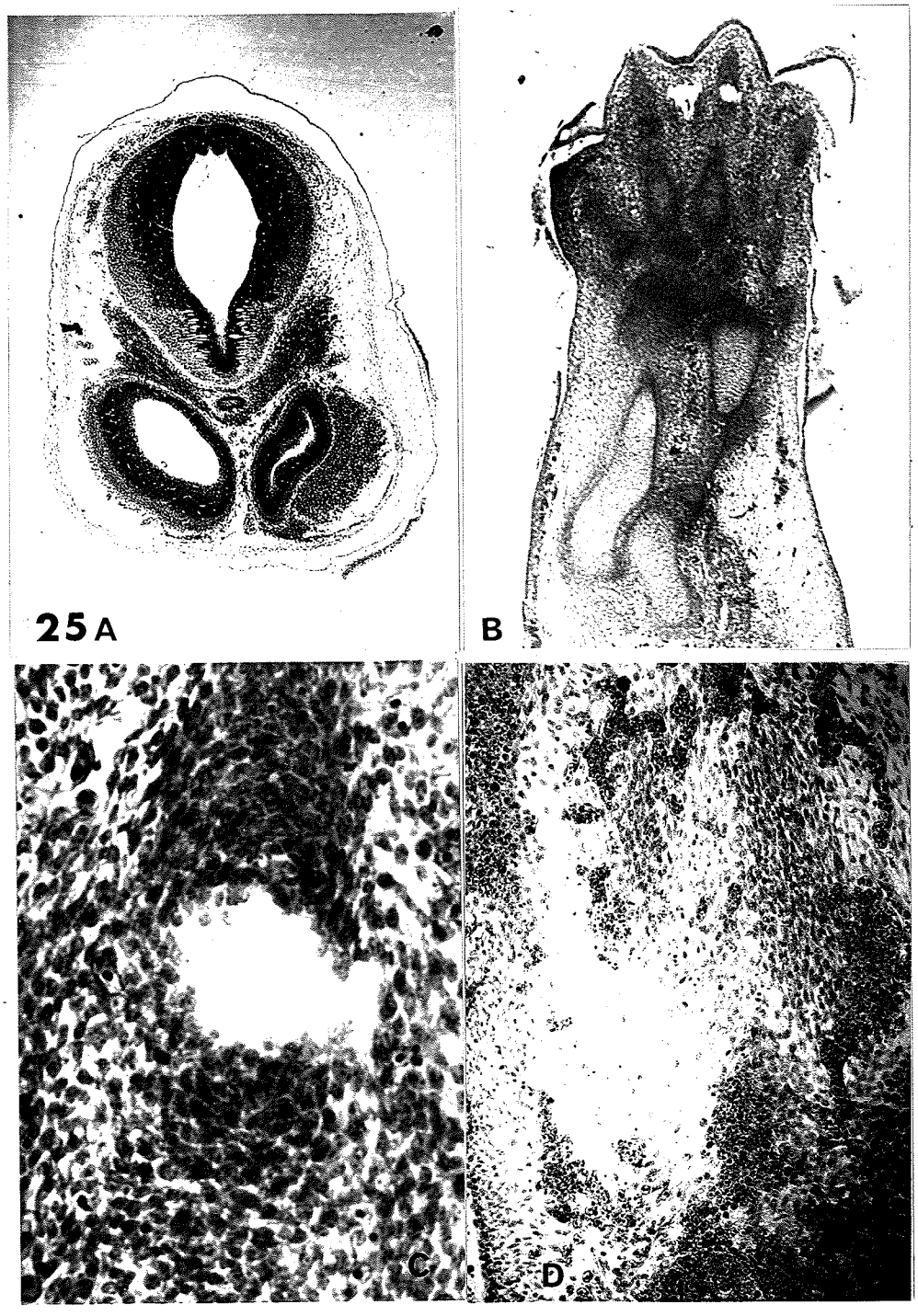
Figure 25. Morphological changes in the head regions and limbs as observed 60 minutes after amniocentesis.

(a) Intra-cranial hemorrhage and separation of connective tissue layers.
(x 15)

(b) Cavitation in phalangeal condensation and deep in the interdigital mesenchyme.
(x 30)

(c) Cavitation in phalangeal condensation as observed in Figure 25b.
(x154)

(d) Focal oedema, cloudy swelling and cellular degeneration, and hemorrhage.
(x 96)


25 A
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D

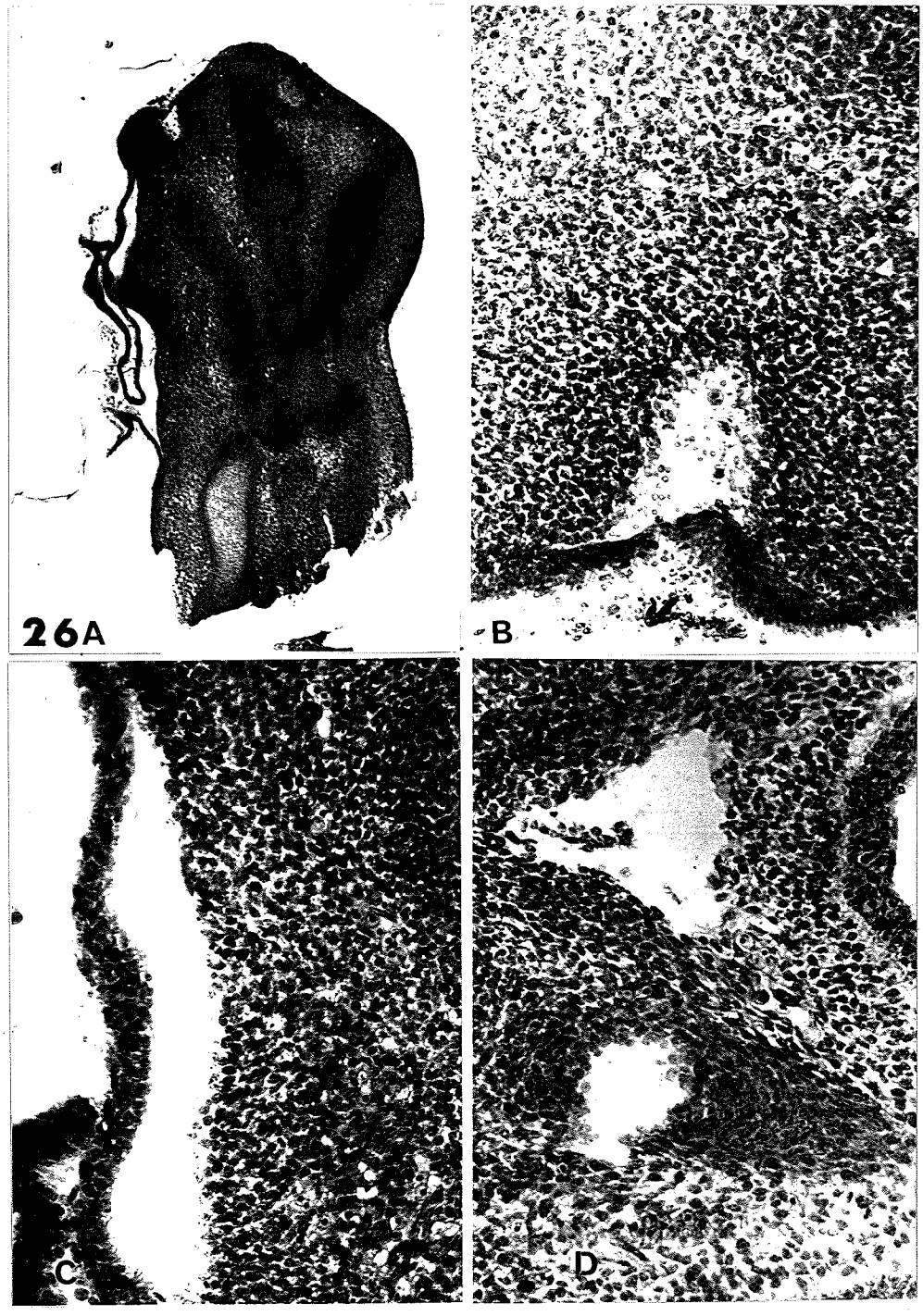
Figure 26. Physiological necrosis and induced necrosis seen at the 60 minute time interval.

(a) Physiological necrosis in the limb of an untreated fetus.
(x 30)

(b) Morphogenetic necrosis in the interdigital mesenchyme.
(x 189)

(c) Sloughing off of epidermis in the extremity of a control fetus.
(x 189)

(d) Necrosis of an extremity induced by amniotic sac puncture.
(x189)


26A
B
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were evident in the limbs and heads of treated fetuses (Figure 27). In addition, in some instances cavitation led to the formation of small superficial blebs on the limbs the floors of which consisted of a reformed epidermal layer (Figure 27b). Some evidence of cell necrosis was seen in the form of cloudy swelling, cell fragmentation, and debris in the interstitial spaces. A few clumps of cells with pyknotic nuclei were found immediately related to the areas of oedema and cavitation.

In the cranial regions of the fetuses studied there was a marked separation of all connective tissue layers (Figures 27 c and d). Large spaces indicative of excessive fluid accumulation were seen in the subcutaneous, intermeningeal and intraventricular areas. Cavitation and necrosis of the nervous tissue itself was also observed and was accompanied by a minor degree of hydrocephalus (Figures 27e and f). Pyknotic nuclei, primitive blood cells, cell debris and interstitial oedema were seen within the cerebral tissue and there appeared to be a reduction in the number of mitotic figures in the ependyma near to the damaged tissue.

At 24 hours the processes of vascular engorgement and disruption, hemorrhage, interstitial oedema and cavitation were observed. In addition, the necrosis and disorganization of several tissues, superficial blebs and the early stages of reductive processes were evident.

There were marked changes in the mesenchymal and cartilagenous pre-skeleton of many limbs (Figure 28). A structural bowing of the long

Figure 27. Lesions observed in fetuses 12 hours after the puncture of the amniotic sac.

(a) Marginal hematoma on the foot.
(x 25)

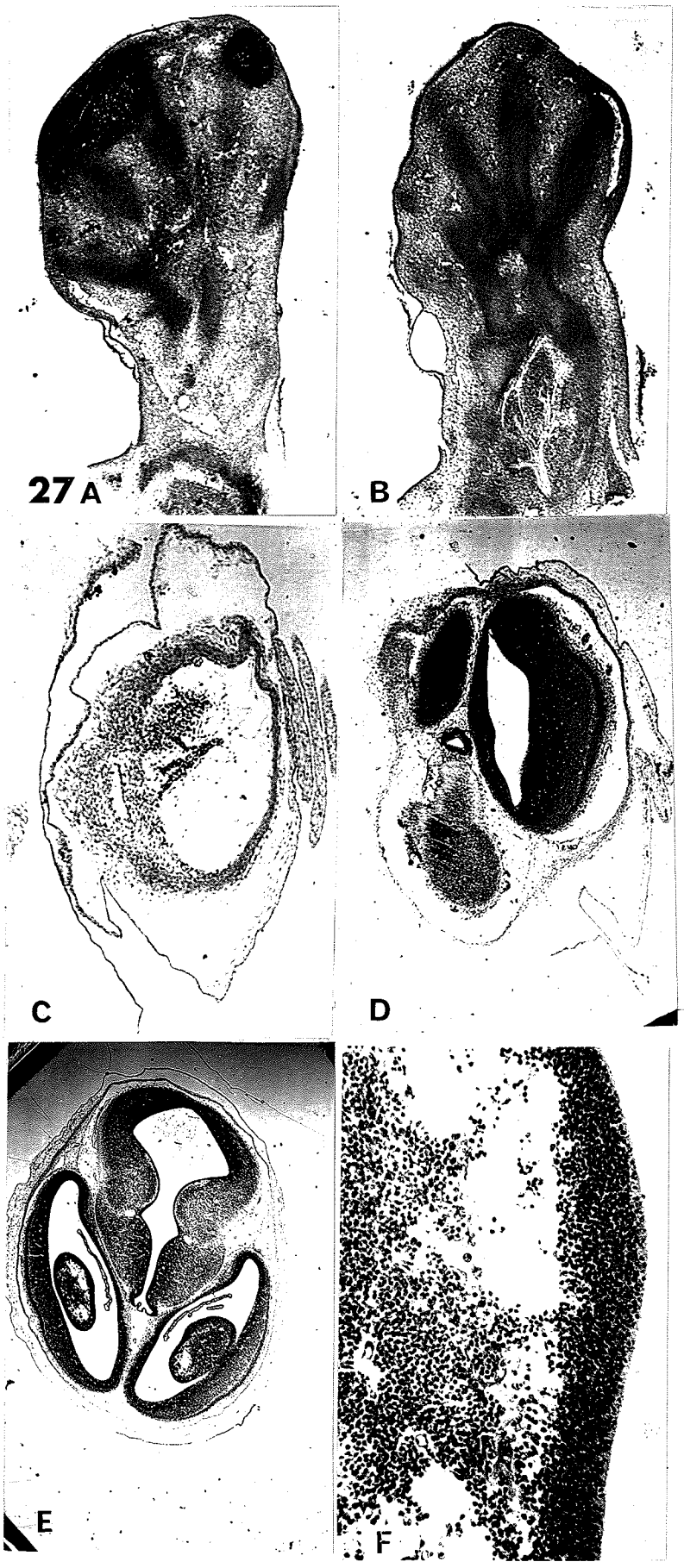
(b) Superficial limb bleb.
(x 25)

(c) Separation of connective tissue layers seen in a cranial bleb.
(x 25)

(d) Changes in the cerebral hemispheres associated with a cranial bleb.
(x 16)

(e) Necrosis and cavitation in the forebrain.
(x 10)

(f) Necrosis in the forebrain.
(x 128)


27 A
B
C
D
E
F

bones of the limb was observed which was accompanied by changes in the organization of the cartilagenous tissue and the presence of chondrocytes in varying stages of degeneration. The chondrocytes lost the closely-packed, layered orientation seen in the long-bones of untreated fetuses (Figure 28c.) and although some lacunae contained apparently viable cells, many contained ghost cells, cells with pyknotic nuclei or peripheral chromatin, or only cell debris (Figure 28d). A large area of necrosis was seen where the carpal primordia are normally present and this was invading the distal epiphysis of the long bone (Figure 28b). Several instances of tissue necrosis and disorganization were also observed in the digital pre-cartilaginous condensations and were found to be associated with severe hemorrhage in the distal margins of the extremities (Figure 28). Necrosis of the phalangeal condensations produced the splitting and the reduction in length of the digital pre-skeleton and destruction of the carpal pre-skeleton and joint spaces.

A process of reduction was consistently and clearly observed in the limbs of treated fetuses and was found to be directly related to the vascular engorgement, hemorrhage and necrosis seen in the fetuses recovered up to and including the 24 hour time interval (Figure 29). Massive marginal hemorrhagic lesions were immediately adjacent to areas of tissue necrosis which was invading both the interdigital mesenchyme and the phalangeal condensations. An epidermal layer separated the distal necrotic portion of the limb which was filled with primitive blood cells, cells with pyknotic nuclei and debris, and eventually

Figure 28. Morphological changes in the cartilaginous pre-skeleton induced in the limbs by amniocentesis as observed in a fetus recovered 24 hours after treatment.

(a) Bowing of the long bones.
(x 24)

(b) Hemorrhage, reductive necrosis and splitting of the phalangeal condensations, interdigital necrosis of the mesenchyme, necrosis of the carpal primordia with invasion into the distal epiphysis of the long bone.
(x 24)

(c) Normal cartilage as observed in a control fetus.
(x 242)

(d) Degeneration of chondrocytes as observed in abnormal limbs.
(x 348)

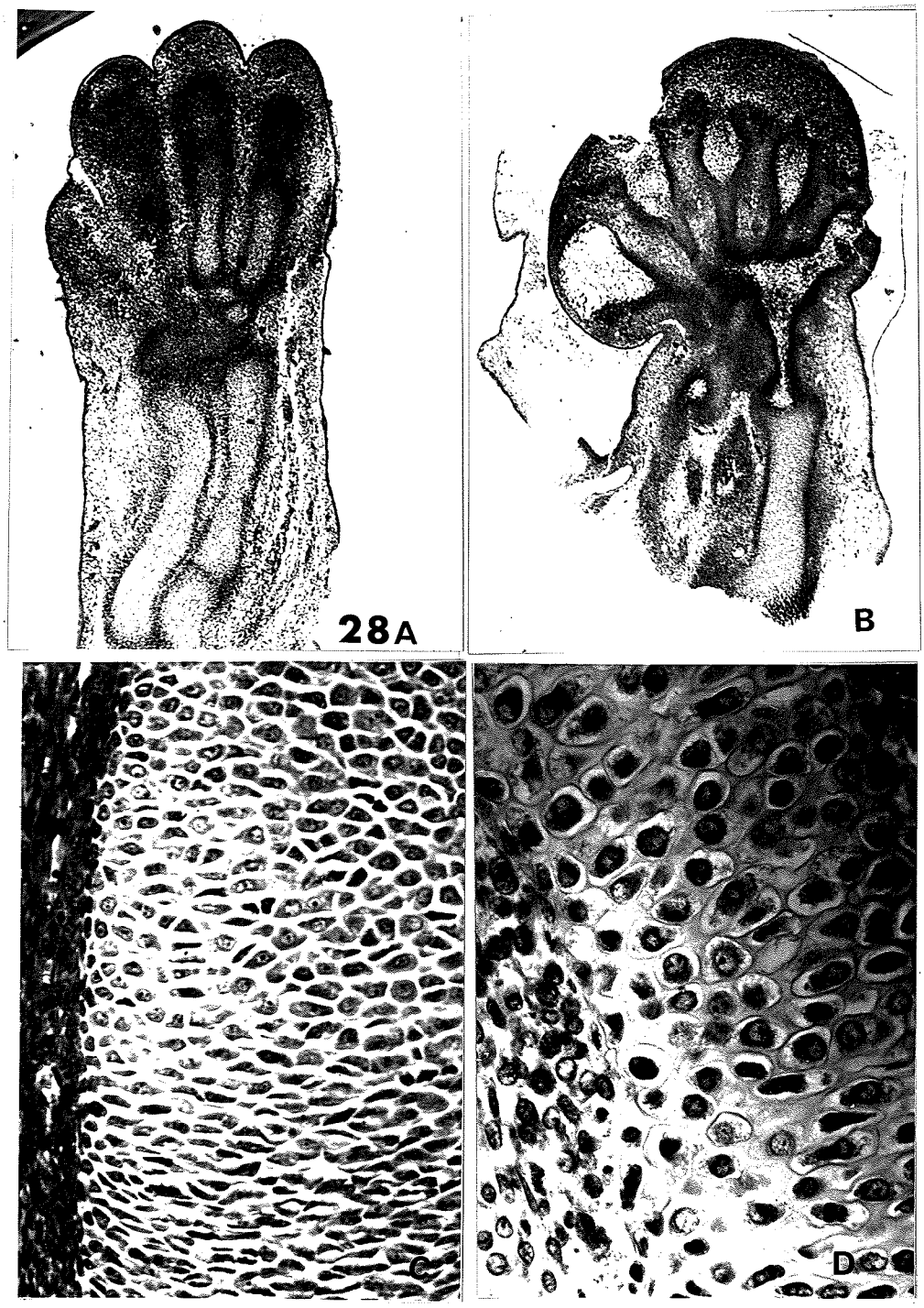

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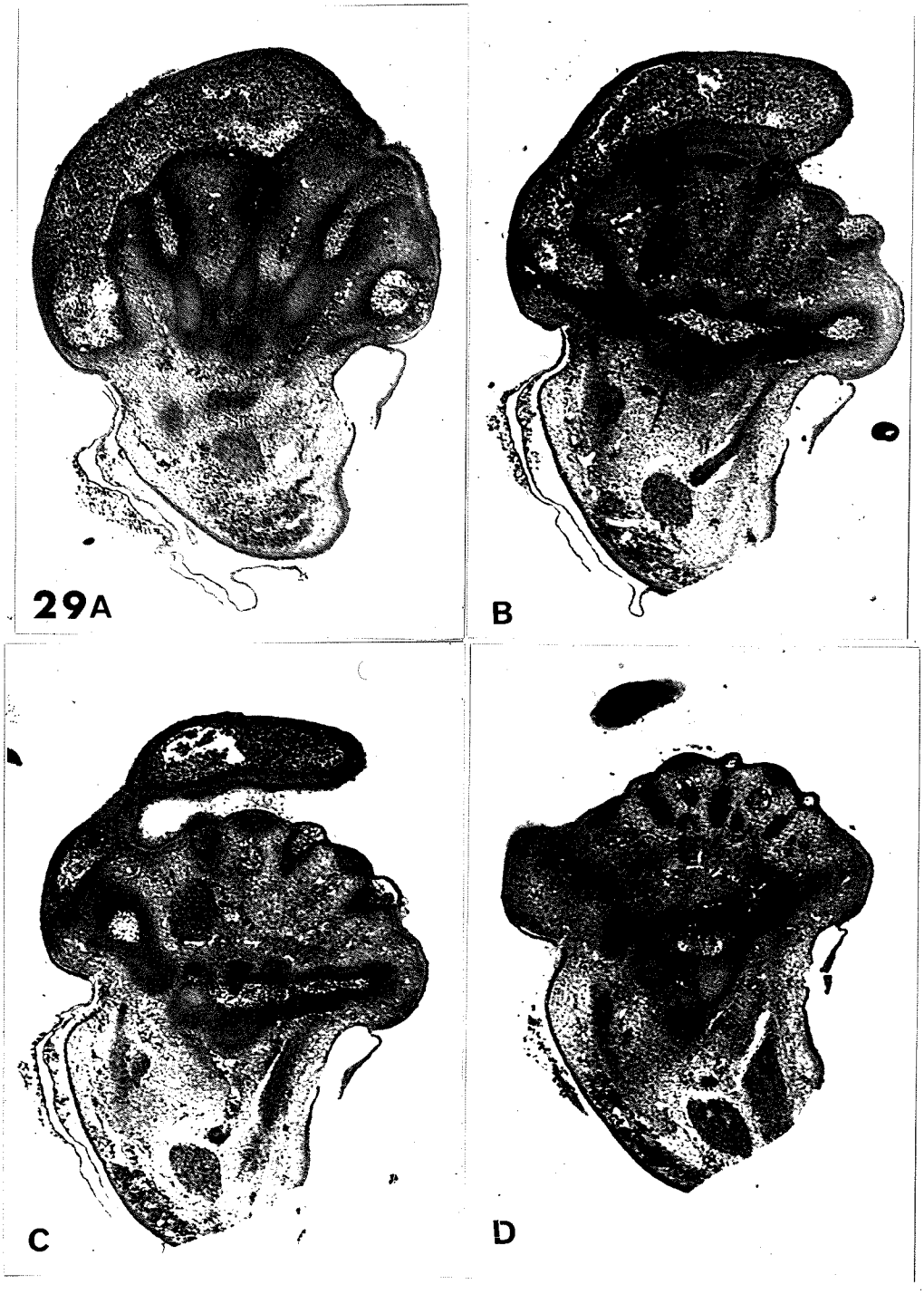
Figure 29. Stages in the progressive reduction of a limb associated with massive marginal hemorrhage as observed in a fetus recovered 24 hours after amniocentesis.
(x 30)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amputated it leaving only a stump of the previously well-formed limb.

A second process of limb reduction was observed which was related to the formation of superficial blebs (Figure 30). The floors of these blebs were in some cases lined continuously with epidermal cells thus forming a well-circumscribed space. The outer epidermal covering of the bleb consisted of ghost cells and cells in varying stages of degeneration. Eventually the bleb appeared to be amputating the distal segment of the limb.

The lesion of the head region described previously as cranioschisis was studied (Figure 31) microscopically. This deformity was found to consist of the herniation of mesenchymal tissue with a large central cavity through the cranium, accompanied at this time interval by the protrusion of cerebral tissue into the cavity. All connective tissue layers of the head were markedly separated, likely by an excessive accumulation of fluid. In contrast the heads recovered at the earlier time intervals showed little if any herniation of cerebral tissue into the mesenchymal protrusion. Serial histological sections revealed gross changes both in the structure of the parts of the brain and in their relationships to each other. In addition, a protrusion of nervous tissue which was filled with engorged blood vessels was seen in the ventricular cavity of the hindbrain (Figure 31b). With the exception of many pyknotic nuclei in the tissue immediately adjacent to this protrusion,

Figure 30. Limb lesions observed 24 hours after amniotic sac puncture.

(a) Superficial limb bleb with partially reformed epidermal floor in association with vascular engorgement, hemorrhage and interstitial oedema of the limb plate. (x 96)

(b) Amputation of the distal limb segment by the coalescence of a superficial bleb. (x 32)

(c) Limb bleb with reformed epidermis in association with marginal hemorrhage, cavitation and necrosis. (x 30)

(d) Marginal surface blebs with engorgement, necrosis and reduction of the distal limb segment. (x 30)

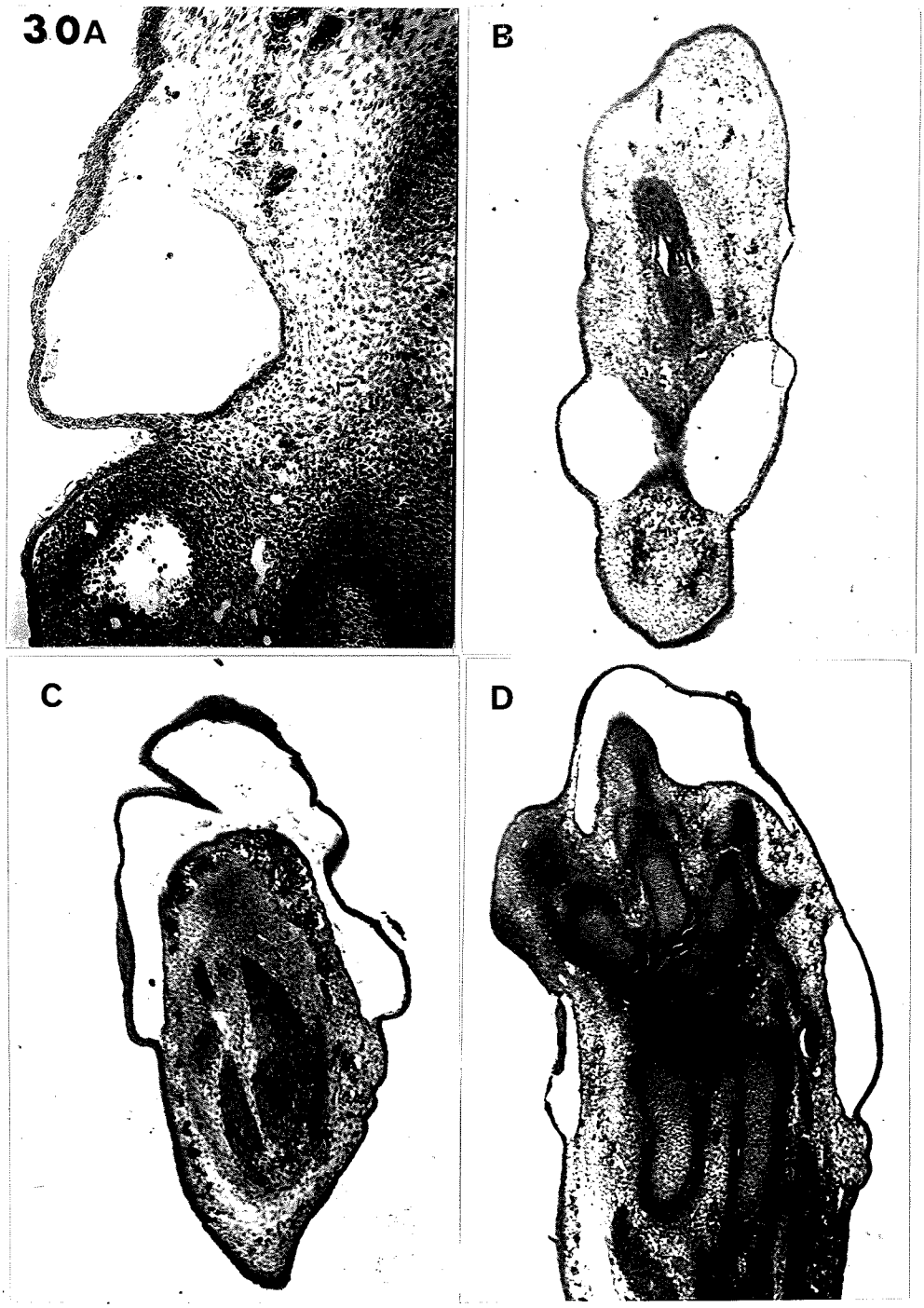
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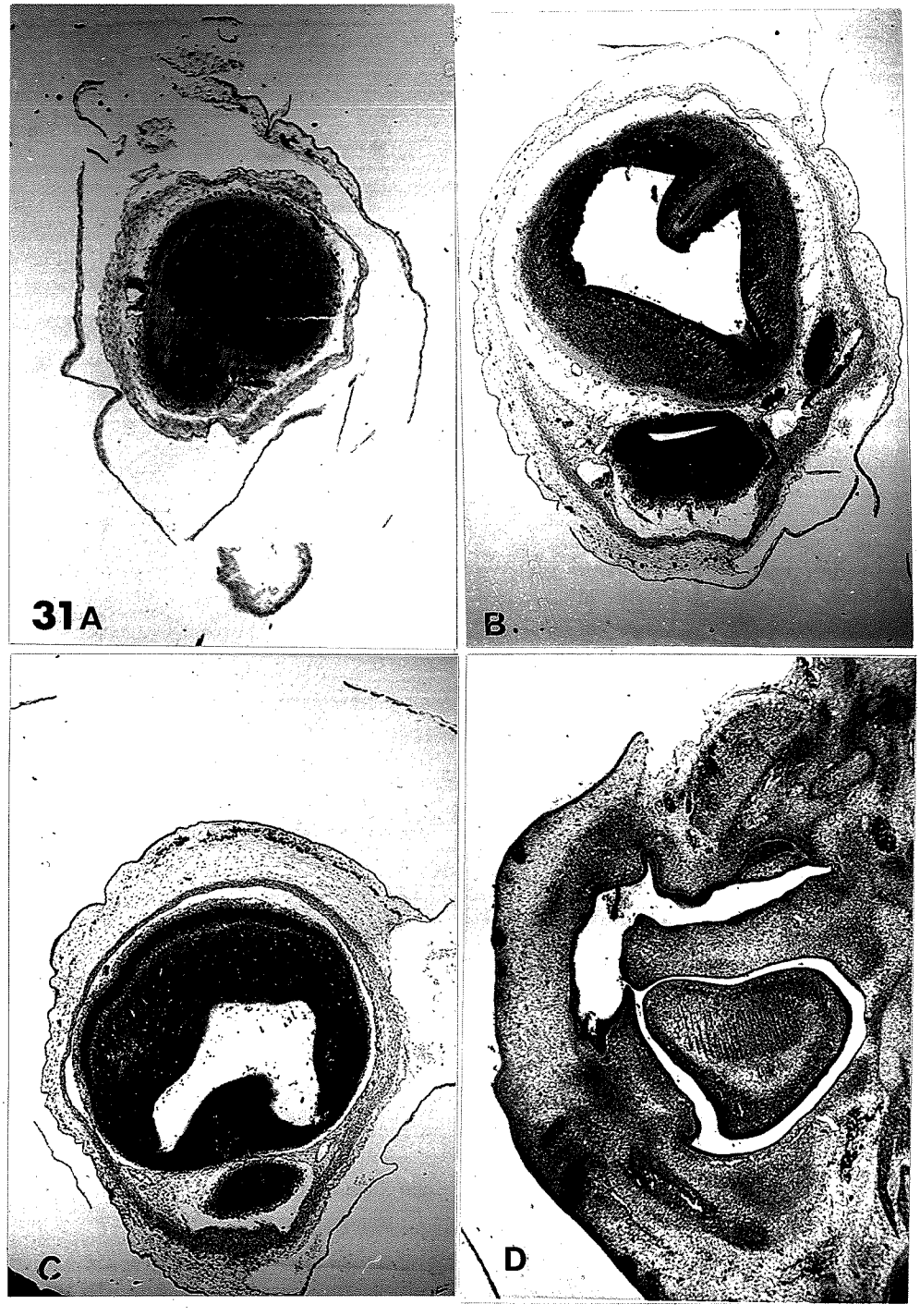
Figure 31. Severe compression in the head region seen 24 hours after amniotic sac puncture.

(a) Herniation of cerebral tissue through the cranium, necrosis and separation of the connective tissue layers.
(x 19)

(b) Protrusion of nervous tissue filled with engorged blood vessels into the ventricular space associated with hydrocephalus of the hindbrain and distortion of the forebrain structure. The spatial relationship between hindbrain and forebrain is grossly abnormal.
(x 15)

(c) Distortion of the cerebral ventricular system.
(x 15)

(d) Palate forced to one side by the tongue.
(x 19)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there were no changes in the cell or tissue morphology of the brain itself. A huge ventricular space was seen in the hindbrain region. In addition, the ventricles of the cerebral hemispheres were grossly distorted (Figure 31c). Further evidence of severe compression of the cranial region was the finding of an intact palate which was forced aside by the tongue (Figure 31d).

The microscopic changes seen in the heads recovered 36 hours following the puncture of the amniotic sac involved both the mesenchymal and the nervous tissues. In addition to the engorgement of subcutaneous vessels and the necrosis and separation of connective tissue layers seen at earlier time intervals there was evidence of necrosis within the nervous tissue (Figure 32). Cells with pyknotic nuclei or peripheral chromatin predominated and many ghost cells were seen. In the outermost layer of nervous tissue which is normally primarily fibrous, only debris was present in the spaces between nuclei (Figure 32b).

Lesions of the limbs exhibited the same pattern as was described for earlier time intervals (Figure 34). Large superficial blebs bounded by engorged and disrupted blood vessels, and necrotic tissue were found. These coalesced and appeared to be amputating or constricting the segment of the limb distal to it. Necrotic reductions associated with hemorrhagic lesions were also observed and there were large areas of necrotic tissue present in all limbs studied (Figure 33).

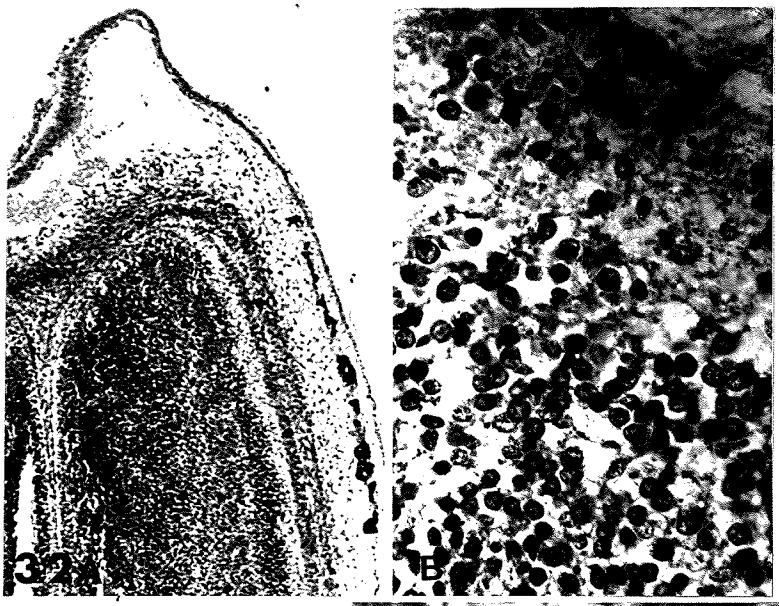
The microscopic changes seen in the heads of experimental fetuses recovered 48 hours after treatment were similar to those described in the earlier time intervals namely, marked separation of all connective

Figure 32. Morphological changes in the head region observed 36 hours after amniotic sac puncture.

(a) Separation of connective tissue layers on the posterior aspect of the head.
(x 25)

(b) Degeneration of the nervous tissue.
(x 409)

Figure 33. Tissue necrosis as observed in the limb of a fetus recovered 48 hours after amniocentesis. Note the presence of cells with pyknotic nuclei, peripheral chromatin and vesiculated cytoplasm, as well as ghost cells and the abundance of debris.
(x 945)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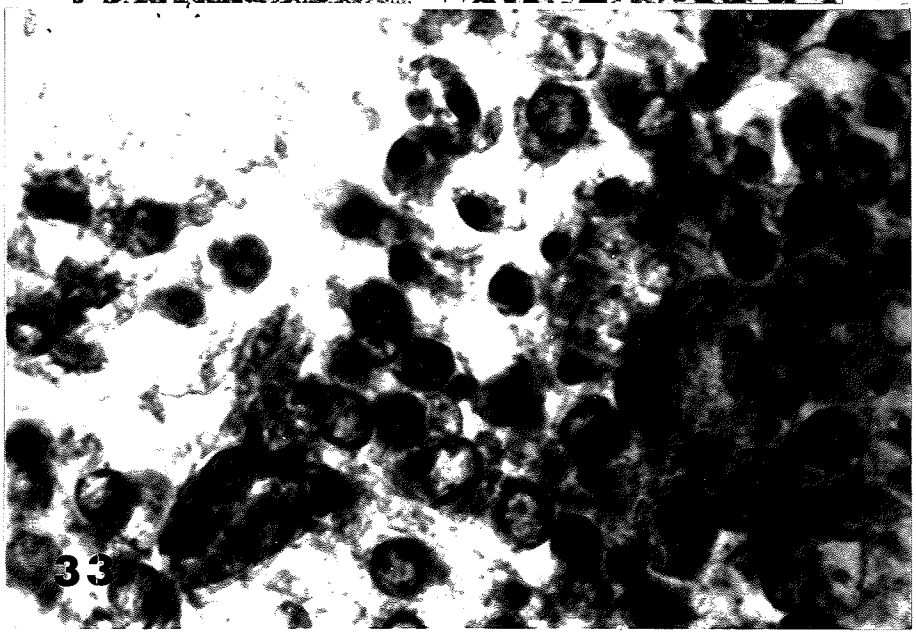
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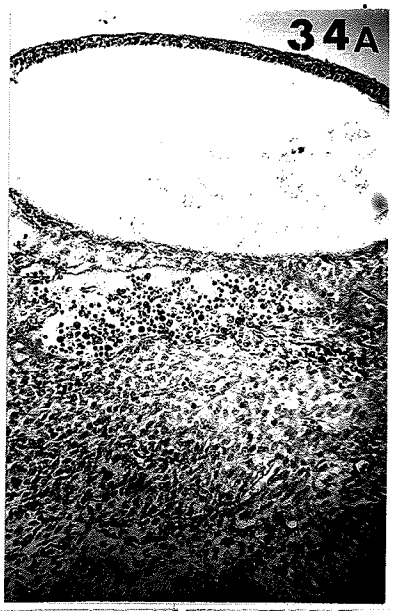
Figure 34. Microscopic changes in the limbs of fetuses recovered 36 hours after amniocentesis.

(a) Dilation of superficial blood vessels, reformed epidermis around the surface bleb and evidence of necrosis.
(x 80)

(b) Vascular disruption associated with necrosis.
(x 252)

(c) Cell necrosis and fragmentation.
(x 252)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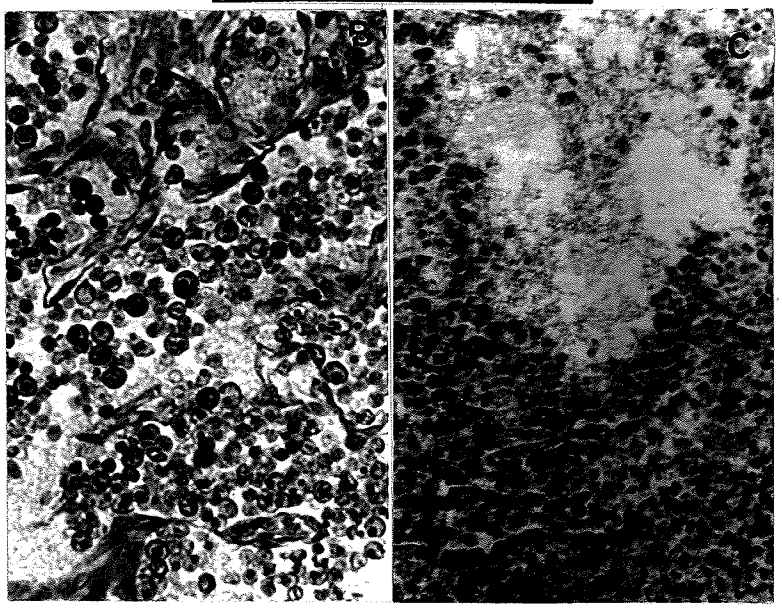
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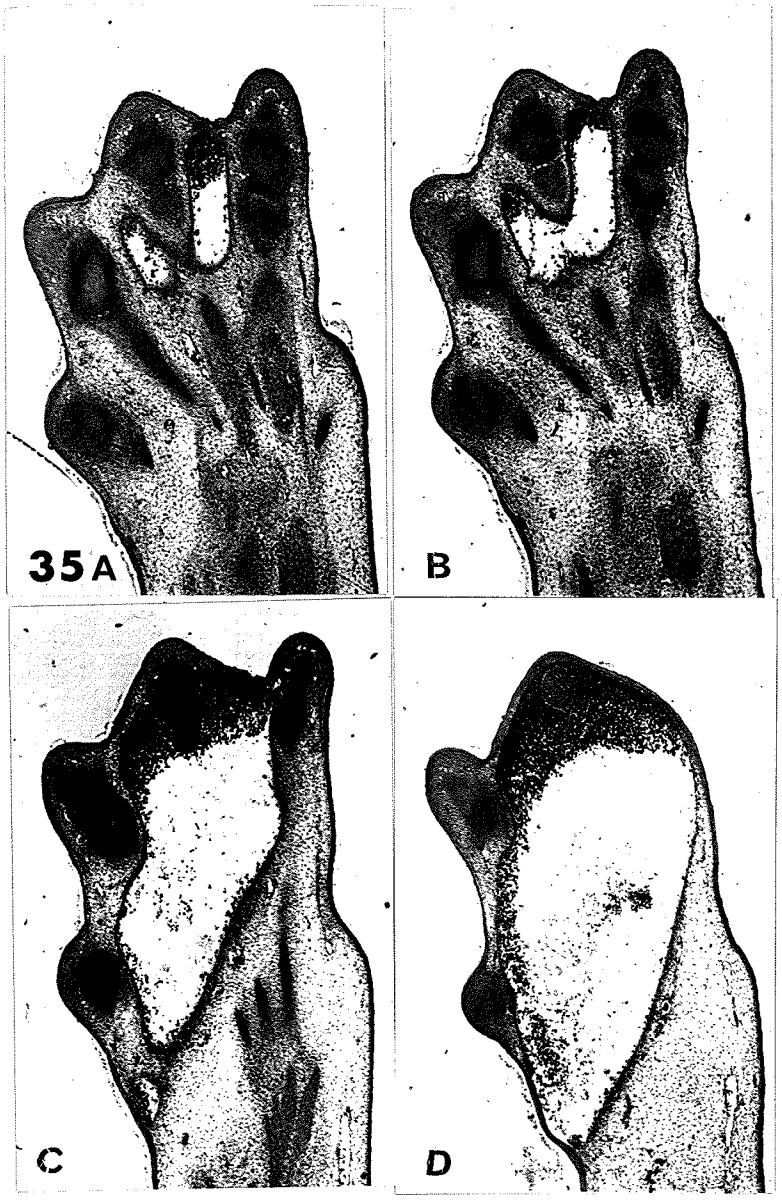
tissue layers, interstitial oedema, slight hydrocehpalus, vascular engorgement, hemorrhage and degeneration of the connective tissues. No changes were detected in the nervous tissue of the heads studied.

The regressive processes leading to the limb reductions/amputations seen on day 20 in The Control of Teratogenicity experiments were evident 48 hours after the puncture of the amniotic sac. The phenomena of vascular engorgement and disruption, hemorrhage, interstitial oedema and cavitation, bleb formation and necrosis were again observed. In addition, the progressive coalescence of large, fluid-filled , clearly defined vacuoles or cavitiesin the interdigital areas, leading to the amputation of each digit in turn was observed (Figure 35). Only a stump of the original limb remained, a malformation which was previously defined as a digital bleb or, adactyly in its later form.

Figure 35. Progressive stages in the reduction of a limb as observed 48 hours after the puncture of the amniotic sac.
(x 25)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SECTIONS I - 4

DISCUSSION

5. DISCUSSION

5.1 PRELIMINARY INVESTIGATION

The direct administration of a test substance to the fetus by injecting it into the amniotic sac is a potentially useful tool in teratological studies (Dostal, 1971, 1973 and Persaud, 1972) and has recently been used by many investigators. It is not clear however, whether the results of such studies can be attributed exclusively to the test substance, or if in fact, the method itself is not at least partially responsible. The Preliminary Investigation was designed to re-assess the usefulness of the intra-amniotic route of administration in teratological studies.

To determine the effects of the surgical procedure alone on the development of the offspring, a group of rats were anaesthetized and subjected to laparotomy. The uterine horns were exteriorized only for the short period of time required to puncture the amniotic sacs, and were subsequently returned to the peritoneal cavity. Since there were no fetal deaths, resorptions or malformations in these litters, and since the fetal measurements of weight, crown-rump length, placental index or length-weight ratio did not differ signficantly from the fetuses of control rats, it was concluded that the surgical procedure alone constitutes no threat to the growth and development of the offspring. This observation is consistent with previous findings (Nicholas, 1962).

The next three treatment schedules investigated the threat to the fetus following puncture of the amniotic membranes and injection of a

given volume of solvent vehicle into the amniotic sac. A paired t-test analysis of fetal weights, crown-rump lengths, placental indices and length-weight ratios revealed no significant differences between treated and untreated fetuses in any of these measures. A mixed analysis of variance however, suggested that there were significant differences between the weights ($p = 0.063$), and between the crown-rump lengths ($p < 0.05$) of treated and untreated offspring.

The differing results of the two statistical procedures may reflect a difference in the sensitivity of the two tests. Both procedures however, were seriously limited by the loss of most of the fetal measurements available for analysis, due to the high mortality rates among treated offspring. The significant differences in fetal weights and in crown-rump lengths found by the analysis of variance are consistent with the findings of severe fetal compression and postural moulding in subsequent experiments of this investigation, with the overall stunting of fetuses subjected to amniotic sac puncture as reported by Persaud (1973), and the hypotheses of Trasler et al. (1956), Persaud (1973) and Singh and Singh (1973), that the underlying mechanism of amniocentesis-induced abnormalities is related to oligohydramnios and compression of the fetus by the contracting uterine musculature.

The most revealing measure of the risk to the fetus following puncturing of the amniotic sac was the incidence of mortality. Since, in the unpunctured fetuses of groups I and II there were no deaths,

resorptions or malformations, the overall fetal mortality (60.4%) among the treated offspring clearly demonstrated that the puncture of the fetal membranes alone was responsible for the high rate of mortality.

The injection into the amniotic sac of 50 µl and 100 µl of distilled water (a possible solvent vehicle) raised the mortality rate still higher. In a study of the teratogenicity of different vehicles, Dostal (1973) found that mortality rates did not differ significantly when 2 µl of 0.45% NaCl, 2 µl of distilled water, or 2 µl and 10 µl of physiological saline was injected into the amniotic sac. In the present experiment, the injection of 50 µl of distilled water produced 100% and 88.9% fetal mortality rates when administered on days 14 and 15 of gestation respectively, and a 100 µl injection resulted in a 100% fetal mortality for both days of treatment. The large volumes injected in these experiments produced a much higher fetal mortality rate than did the 10 µl and 2 µl injections reported by Dostal (1973). The high incidence of fetal mortality following amniotic sac puncture on gestational day 14 seen in this study (76.5%) in comparison to the low incidence reported for the same gestational day by Dostal (1973) is likely related to the different calibre of the instrument used to make the punctures in the two studies, and will be discussed later in more detail (see page 89, The Control of Teratogenicity).

Mortality rates were found to be related to the gestational day on which the amniotic sac was punctured. The incidence of fetal deaths for all treatments combined were high on each of gestational days 14 (92.2%)

and 15 (86.8%), but fell to 26.1% on day 16. This reduction in fetal mortality with increasing gestational age reflects the reduction of "fetal plasticity" (Dunn, 1974), or the increased ability of the fetus to resist damage, with advancing maturity.

5.2 CONTROL OF TERATOGENICITY

The Experiments were designed to investigate the possibility of refining the intra-amniotic technique for use in teratological studies a) by reducing the size of the puncture site and b) by attempting to close the puncture site, thereby reducing or preventing the leakage of amniotic fluid.

The paired t-test analysis for several fetal parameters, including the weights, crown-rump lengths, placental indices and length-weight ratios revealed no consistent pattern of variation between treated and untreated offspring for any of the treatment schedules. However, where a significant difference was found to exist, it was always related to the measurements of weight, length or weight-length ratio. It appears therefore that a compression mechanism, as previously discussed, is involved in these changes, but that this compression phenomenon has a variable effect on different litters as well as on individual fetuses within each litter.

The mixed analysis of variance revealed that among the experimental offspring of the different treatment schedules, there were no significant differences in any of the fetal measurements observed. There were however,

highly significant differences in the weights, crown-rump lengths and length-weight ratios between treated and untreated fetuses for all treatment schedules. It can be concluded therefore that all treatment schedules (amniotic sac puncture with either 26, 30 or 34 g needle or puncturing with a 26 g needle plus a seal) did in fact, produce significant changes in fetal growth and development. These changes however, did not differ relative to the calibre of the needle nor to the attempt to close the puncture sites. The fact that there were no significant differences between placental indices of treated and untreated fetuses, revealed that the fetal weight changes were accompanied by similar placental weight changes; the ratio therefore remained relatively constant.

That the effects of amniotic sac puncture on fetal weights and crown-rump lengths were group-dependent, i.e. each litter seems capable of responding differently to the same treatment, can be seen in the highly significant interaction factor ($p < 0.05$) for the length-weight ratio. This means that although the difference in this ratio were significant between treated and untreated fetuses, the manner in which they varied was different in the different treatment groups. Perhaps this differental response of the fetuses of individual litters to the same treatment can be explained by the size of the litter. It is conceivable that as the number of offspring in a given uterine horn increases, the severity of the compression to which each fetus is subjected as the uterus contracts, would also increase.

The different results from the paired t-test analysis and the mixed analysis of variance likely reflects a difference in the sensitivity of these statistical tests as well as the limited number of measurements, due to the deaths of many experimental offspring, available for analysis.

Again, fetal mortality was specific for the different treatment schedules. With the reduction in the needle calibre from 26, to 30, to 34, and concomitant reduction in the incidence of fetal death (28.26%, 16.67% and 13.04% respectively) was observed. When the Gelfoam seal was applied to the puncture site made with a 26 g needle (group IV), fetal mortality was reduced from 28.26%, seen among fetuses with the unsealed puncture, to 14.81%. In this instance the seal was associated with a reduction in the fetal mortality to a level not markedly different from that observed among fetuses exposed to punctures with 30 and 34 g needles. However, when both uterine horns were punctured, either with a 26 or 34 g needle, and the seal applied to the puncture sites of one side only, the incidence of fetal deaths was higher in the uterine horns for which closure of the puncture sites was attempted. Also, when mortality and morbidity rates were combined, the offspring whose sacs were punctured with a 26 g needle and to which Gelfoam was applied, showed the highest rates among all groups. These latter observations contradict the initial observation that the seal ameliorated the deleterious effects on the fetus induced by puncturing the amniotic sac.

When the incidences of fetal mortality and morbidity were combined

only one trend remained evident, that being the reduction of the teratogenic effect of amniocentesis when the puncture was made with a 34 g needle.

5.3 MORPHOGENESIS OF DEVELOPMENTAL DEFECTS FOLLOWING AMNIOTIC SAC PUNCTURE

Prior to this study, the descriptions of malformations induced in experimental animals following puncture of the amniotic sac have focused on the well-formed lesions found in fetuses recovered four to six days after treatment i.e. near the end of the normal gestational period.

The abnormalities detected in the offspring have related primarily to the head, limbs and abdominal wall. Cleft palate, microstomia, micrognathia, exencephaly/exencephalocoele have been described in the head region. Anomalies of the limbs have included meromelia, adactyly, syndactyly, malrotations, club foot, constriction rings and amputations. Short umbilical cords, abnormalities of the abdominal wall, have been observed in the abdominal region; short thick trunks and stunting, as well as hemorrhagic lesions in the extremities have been consistently reported (Trasler et al., 1956; Kendrick and Feild, 1967; De Meyer and Baird, 1969; Poswillo and Sopher, 1971; Kino, 1972; Love and Vickers, 1972; Persaud, 1973, Singh and Singh, 1973, 1974; and Singh et al., 1974). In the period between treatment and observation however, many undefined processes of damage and repair are likely to have occurred which resulted in the final anomaly.

Love and Vickers (1973) studied the microscopic morphology of limb

malformations present in rat fetuses recovered six days after amniocentesis, at the end of gestation. They found numerous subcutaneous hemorrhages which sometimes took the form of blebs, as well as impaired endosteal, periosteal and enchochondral osteogenesis. Kino (1972) studied the morphogenesis of amniocentesis-induced amputations and reductions in rats, and Singh and Singh (1973) using the dissecting microscope observed hemorrhagic lesions between the digits of fetal rats as early as five minutes after amniocentesis and the withdrawl of amniotic fluid.

The findings of the present investigation with respect to the gross and microscopic changes present in rat fetuses following amniocentesis, are in agreement with the observations of other investigators (Persaud, 1972; Kino, 1972; Singh and Singh, 1973, 1974; Singh et al., 1974). In addition, the present study provides further details of cellular and tissue changes from 15 minutes to 48 hours after amniocentesis, and a working hypothesis as to the mechanisms underlying these is advanced.

Two types of cell death are particularly significant to investigators of teratogenic mechanisms (Faber, 1971; Menkes et al., 1970; and Schweichel and Merker, 1973). Physiological or morphogenetic necrosis is a normal embryological process and reflects a process of programmed cell death which is essential to the fine sculpturing of many organs. It is essential to distinguish between this type of necrosis and that which is experimentally-induced. Although there are many parallels, several morphological differences at the light microscopic level were

evident in the present investigation. Morphogenetic necrosis was observed in the interdigital mesenchyme immediately below the epidermis and in the sloughing off of epidermal layers in the limbs of treated fetuses. Necrosis induced by amniocentesis was detected deep in the interdigital mesenchyme and in the cartilagenous and mesenchymal pre-skeleton of fetal limbs. These necrotic areas were immediately associated with vascular changes and changes in the degree of contiguity between the cells and tissue layers in the surrounding tissues which were not present in the areas of morphogenetic necrosis.

The engorgement, dilatation and disruption of the blood vessels of the limbs and heads of treated fetuses, and massive hemorrhage into the surrounding tissues were consistently and clearly detected from 15 minutes to 48 hours following puncture of the amniotic sac. In the earliest time intervals (15 and 30 minutes), only hypervolaemia and hemorrhage were detected. This was interpreted as representing a state of severe venous stasis or congestion which could have been produced a) by compression of the umbilical cord and placenta by the fetus as a result of uterine contraction, b) by constriction of the placenta alone as the myometrium contracted following the leakage of amniotic fluid, or c) by the combined effect of umbilical compression and placental constriction. It is suggested that purely circumstantial factors such as the physical posture or spatial orientation of the fetus at the time of puncture would determine the mechanism involved. This hypothesis is supported by the severe pooling of blood in the umbilical cord, in the flattened umbilical

cords and abdominal walls, in the reduction or absence of the normal herniation of the gut into the umbilical cord and in the examples of postural moulding suggestive of compression, which were frequently detected in fetuses with hemorrhagic lesions.

Venous stasis in the superficial blood vessels, caused by direct pressure from the contracting uterus, could also explain both the generalized and localized subcutaneous hemorrhages seen in relation to the spinal column, on the snout and on the outer aspects of the joints of the extremities. It would also explain the higher frequency and greater severity of hind limb than forelimb malformation reported by several investigators (Singh and Singh, 1973; Love and Vickers, 1972), since the large overhanging fetal head would protect the smaller forelimbs from the contracting uterus. Whether the source of venous stasis is peripheral or central, the fragile embryonic vessels would inevitably rupture. This engorgement, rupture and hemorrhage was the only lesion seen at 15 and 30 minutes and has been reported to occur as early as five minutes after amniocentesis by Singh and Singh (1973).

Sixty minutes after treatment, foci of interstitial oedema were evident. This oedema could have been caused by the vascular damage, but it was frequently seen in tissues quite distant from the hemorrhagic lesions. Therefore, a second mechanism, fetal hypoxia, is proposed to be acting by this time.

Grabowski (1970) discussed embryonic oxygen deficiency as a non-specific teratogen and reported cranioschisis, encephalocoele, hydrocephaly, cleft lip and palate, micrognathy, umbilical herniation, neural tube and

brain defects, amelia and other limb abnormalities, as well as severe growth retardation to be among the many malformations associated with fetal hypoxia. Leist and Grauwiler (1974) described the fetal pathology in rats following clamping of the major uterine vessels on day 14 of gestation. They described oedema in the interstitial mesenchyme of the limb plates together with marked dilation of the fetal vessels, necrotic changes, large blisters and hematomas, skeletal retardation, bilateral limb anomalies and cleft palate in fetuses following variable periods of clamping. These changes were attributed to fetal hypoxia and malnutrition during the period of clamping.

The effects of hypoxia as described by these investigators parallel the morphological changes observed in the present study of fetuses subjected to amniotic sac puncture. On the basis of the morphological changes observed, two mechanisms are proposed by which the limb reductions could be induced by amniocentesis. The first is initiated immediately after amniocentesis as either general or local venous stasis. Rupture of the engorged peripheral blood vessels produces hemorrhagic lesions in the surrounding tissue within minutes after treatment. By 60 minutes the vascular changes have probably been followed by the second mechanism, fetal hypoxia.

Embryonic oxygen deficiency can occur secondary to the obstruction of the feto-maternal circulation or to pressure applied to local blood channels. Morphological evidences of hypoxia become manifest microscopically as early as 60 minutes after treatment and take the form of interstitial

oedema and the excessive accumulation of fluid leading to the separation of tissue layers or cavitation. This fluid, consisting of blood plasma and eventually blood proteins, leaks from the hypoxia-damaged endothelium. Further endothelial degeneration results in the release of primitve blood cells into the surrounding tissues without the formation of massive hematomas. Ultimately the increase of interstitial eodema results in the formation of superficial blebs on the limbs, the separation of tissue layers in the head regions and the cavities or vacuoles which were detected in the interdigital mesenchymal tissue which coalesced and appeared to amputate the segments of the limb distal to it. These two pathways explain the morphological variability in the reductive lesions observed in the limbs of treated fetuses. Both pathways ultimately involve embryonic oxygen deficiency as the primary teratogen.

The lesions observed in the head regions can be explained by mechanical compression and/or hypoxia. It is postulated that an excessive accumulation of fluid in the head, leading to the separation of connective tissue layers and ultimately to the necrosis of mesenchymal and nervous tissues, initially was caused by the rupture of blood vessels and only later from hypoxic damage to the endothelium. The hydrocephalus and cranioschisis observed was likely caused by uterine pressure applied to some point(s) of the spinal column and thereby obstructing the normal circulation of the cerebrospinal fluid. Hypoxia or direct pressure could explain the necrosis seen in the nervous tissue itself.

On the basis of the present investigation, the following working hypothesis relating to the morphogenesis of the developmental defects following amniotic sac puncture is proposed:

a) Puncture of the amniotic sac leads to the loss of amniotic fluid and allows the contracting uterus to compress and immobilize the fetus.

b) The compression by the uterus may result in any one or combination of the following: the interruption of the feto-maternal circulation producing severe, general venous stasis; pressure on the fetal epidermis resulting in local or generalized subcutaneous hemorrhages; the obstruction of normal cerebrospinal fluid circulation; the upward herniation of the central nervous system resulting in cranioschisis; postural moulding.

Where venous stasis results from amniocentesis, the initial vascular engorgement causes the early rupture of the fragile fetal blood vessels and the massive hematomas observed in the heads and limbs of treated fetuses. Ultimately the venous stasis or congestion, if it persists, results in embryonic oxygen deficiency.

Hypoxic degeneration of the endothelium probably causes the observed interstitial oedema, which in turn leads to tissue cavitation, separation of tissue layers and formation of superficial blebs in the extremities, and the oedema frequently observed. Thus, hypoxia is likely ultimately responsible for the necrotic changes in the various tissues studied.

Depending on the severity of the compression and the duration of the hypoxia, the fetus may die or may initiate repair processes, such as the regeneration of epidermal surfaces between necrotic and viable tissues, as observed in some fetuses.

5.4 CLINICAL AMNIOCENTESIS

The recent use of amniocentesis in clinical practice for prenatal diagnosis and as a tool in experimental teratology has stimulated considerable concern regarding the possible adverse effects of the procedure on the offspring of both humans (Dewhurst, 1956; Aladjem, 1969) and experimental animals (Trasler et al., 1956; Poswillo, 1972). Despite the abundance of reports describing the malformations typically induced in experimental animals, little serious attention has been given to the implications of these studies in clinical practice.

Nadler (1970), Nadler and Gerbie (1971) and Hamerton et al. (1974) caution against the indiscriminate use of amniocentesis in humans on the basis of causing possible harm to both the mother and the fetus.

Valenti (cited in Aladjem, 1969) reported three cases of club foot in the offspring of women monitored by amniocentesis and expressed concern about the lack of detailed follow-up studies. Creaseman et al. (1968) alerted those using amniocentesis to the complications involved. In a survey of the available case reports he noted that severe fetal trauma (hemothorax, lacerated myocardium, retained catheter tip, peritonitis, liver damage, sepsis and needle injuries to the brain) had been reported in as high as 11% of the offspring of treated mothers. Trauma to the placenta with increased iso-immunization and severe fetal anemia and

shock leading to intra-uterine death were discussed and sometimes the incidence of feto-maternal hemorrhage was reported to be as high as 10%. Hanid (1975) reported a case of pneumothorax and surgical emphysema in a newborn baby which was caused by amniocentesis.

The reports available deal primarily with fetal death or morbidity resulting from trauma. There is only one mention of the postural moulding (Aladjem, 1969) suggestive of oligohydramnios or intra-uterine compression which has been consistently observed in experimental studies.

Greater attention should be given to both the statistical incidence of fetal or placental trauma and to the deformities associated with compression and with oligohydramnios. Until such a comprehensive follow-up report is available, it cannot be conclusively accepted that clinical amniocentesis has 1% or less incidence of complications.

CONCLUSIONS

CONCLUSIONS

The following conclusions were made from the results of the Preliminary Investigation:

a) Amniocentesis on gestational days 14, 15 or 16 in the rat produced a high incidence of mortality and a significant reduction in the weights and crown-rump lengths of treated offspring.

b) With increasing gestational age at the time of treatment, the incidence of fetal mortality decreased.

c) By minimizing the volume of the solvent vehicle to be injected the incidence of fetal mortality can be reduced.

The attempt to reduce the teratogenicity of puncturing the amniotic sac revealed;

a) that the deleterious effects of amniocentesis on the fetal measurements of weight, crown-rump length and length-weight ratio were not ameliorated either by reducing the calibre of the needle used to make the puncture or by applying a Gelfoam seal to the puncture sites,

b) that the incidence of fetal mortality, but not the combined incidence of fetal morbidity and mortality, was reduced as the calibre of the needle used to make the puncture was reduced,

c) that the evidence regarding the ability of the Gelfoam seal to ameliorate the deleterious effects of amniocentesis is conflicting and inconclusive.

Studies on the morphogenesis of developmental defects following amniotic sac puncture led to the following conclusions:

a) The observed hemorrhagic lesions may have resulted from hypervolaemia and/or hypoxia in the fetus, secondary to compressive changes which occurred in utero .

b) The final reductive lesions and amputations of the limbs represent the advanced stages of several processes of necrosis and repair subsequent to hypervolaemia and/or hypoxia.

c) The compression of the fetus and the obstruction of the feto-maternal circulation by the uterus, secondary to amniocentesis and the resultant oligohydramnios, is the primary aetiological factor in this syndrome.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abramovich, D.R. (1973). The volume of amniotic fluid and factors affecting or regulating this. In, Amniotic Fluid: Research and Clinical Application (eds. Fairweather, D.V.I. and Eskes, T.K.A.B.). Exerpta Medica, Amsterdam, pp. 29-51.

Aladjem, S. (1969). Artificially induced variations of amniotic fluid volume in early pregnancy. In transcript from, 5th Rochester Trophoblast Conference (eds. Lund, C.T. and Choate, J.W.). Unpublished.

Arey, C.B. (1965). Developmental Anatomy. A textbook and laboratory manual of embryology. W.B. Saunders Co., Philadelphia.

Baker, C.J. and Rudolph, A.J. (1971). Congenital ring constrictions and intra-uterine amputations. Am. J. Dis. Child. 121, 393-400.

Behrman, R.E., Parker, J.T. and De Lannoy Jr., C.W. (1967). Placental growth and the formation of the amniotic fluid. Nature (Lond.) 214, 678-680.

Bergstrom, S. (1971). Surface ultrastructure of human amnion and chorion in early pregnancy. A scanning electron microscope study. Obst. and Gynec. 38, 513-524.

Blanc, W.A. (1970). Amniotic band syndrome. Am. J. Pathol. 59, 27a.

Blanc, W.A., Mattison, D.R., Kane, R. and Chauhan, P. (1971). L.S.D., intra-uterine amputation, and amniotic band syndrome. Lancet 2, 158-159.

Blystead, W., Landing, B.W. and Smith, C.A. (1951). Pulmonary hyaline membranes in newborn infants. Pediatrics 8, 5-21.

Bourne, G.L. (1960). The microscopic anatomy of the human amnion and chorion. Am. J. Obstet. Gynec. 79, 1070-1073.

Bourne, G.L. (1962). The Human Amnion and Chorion. Lloyd-Duke, London, pp. 12-24, 25-46, 196-213.

Bourne, G.L. and Lacy, D. (1960). Ultrastructure of human amnion and its possible relation to the circulation of amniotic fluid. Nature (Lond.) 126, 952-954.

Boyd, J.D. and Hughes, A.F.W. (1954). Observations on human chorionic villi using the electron microscope. J. Anat. (Lond.) 88, 356-362.

Browne, D. (1955). Congenital deformities of mechanical origin. Arch. Dis. Child. 30, 37-41.

Browne, D. (1967). A mechanistic interpretation of certain malformations. In, Advances in Teratology 2 (ed. Woollam, D.H.M.). Logos Press, London, pp. 12-36.

Browne, F.J. (1924). On the weight and length of normal foetuses and the weights of foetal organs based on a series of 218 selected cases in Edinburgh. In, Child life Investigations, Medical Research Council, London.

Creaseman, W.T., Lawrence, R.A. and Thiede, H.A. (1968). Fetal complications of amniocentesis. J.A.M.A. 204, 949-952.

Cruickshank, J.N. and Miller, M.J. (1923). The weight of fetal organs. A study of the relations between organ weight and body weight in the later months of development based upon the examination of 470 normal foetuses out of a series of 1,000 foetuses and new-born infants in Glasgow. In, Child Life Investigations, Medical Research Council, London, 1924.

Danforth, D.N. and Hull, R.W. (1958). The microscopic anatomy of fetal membranes with particular reference to the detailed structure of the amnion. Am. J. Obstet. Gynec. 15, 536-550.

Davies, J. and Robson, J.M. (1970). The effects of vassopressin, adrenaline, and noradrenaline on the mouse foetus. Br. J. Pharmac. 38, 446P.

Davis, M.E. and Potter, E.L. (1957). Congenital malformations and obstetrics. Pediatrics 19, 719-724.

Dawson, A.B. (1926). A note on the staining of cleared specimens with alizarin red S. Stain Tech. 1, 123-124.

De Meyer, W. and Baird, I. (1969). Mortality and skeletal malformations from amniocentesis and oligohydramnios in rats: cleft palate, club foot, microstomia and adactyly. Teratology 2, 33-38.

Dempsey, E.W. and Wislocki, G.B. (1953). Electron microscopy of human placental villi. Anat. Rec. 117, 609.

Dewhurst, C.J. (1956). Diagnosis of sex before birth. Lancet 1, 471-472.

Dostal, M. (1971). Intra-amniotic application of hydrocortisone in mice. Teratology 4, 63-68.

Dostal, M. (1973). Effect of some non-specific factors accompanying intra-amniotic injection in mouse foetus. Acta Morphologica 21.

Drachman, D.B. and Coulombre, A.J. (1962). Experimental club foot and arthrogryposis multiplex congenita. Lancet 2, 523-527.

Dunn, P.M. (1971). Congenital deformation following premature rupture of the membranes. Teratology 4, 487.

Dunn, P. (1974). Congenital postural deformities: Further perinatal associations. Proc. roy. Soc. Med. 67, 1174-1178.

Edwards, J.A. (1968). The external development of the rabbit and rat embryo. In, Advances in Teratology 3 (ed. Woollam, D.M.H.). Logos Press, London, pp. 239-263.

Faber, J. (1971). Vertebrate limb ontongeny and limb regeneration: morphogenetic parallels. Adv. Morphogen. 9, 127-147.

Fairweather, D.V.I. (1973). Techniques and safety of amniocentesis. In, Amniotic Fluid: Research and Clinical Application (ed. Fairweather, D.V.I. and Eskes, T.K.A.B.). Exerpta Medica, Amsterdam, pp. 19-28.

Flexnor, L.B., Cowie, D.B., Hellman, I.M., Wilde, W.S., and Vosburgh, G.J. (1948). The permeability of the human placenta to sodium in normal and abnormal pregnancies and the supply of sodium to the human fetus as determined with radioactive sodium. Amer. J. Obstet. Gynec. 55, 469-480.

Fuchs, F. (1970). Amniocentesis: Techniques and complications. In, Early Diagnosis of Human Genetic Defects (ed. Harris, M.). U.S. Government Printing Office, Washington, D.C., pp. 11-16.

Grabowski, C.T. (1970). Embryonic oxygen deficiency - a physiological approach to analysis of teratological mechanisms. In, Advances in Teratology 4 (ed. Woollam, D.M.H.). Logos Press, London, pp. 123-167.

Gray, M.J., Nelsen, E.D. and Plenti, A.A. (1956). Estimation of water transfer from amniotic fluid to fetus. Proc. Soc. exp. Biol. (N.Y.) 92, 463-464.

Gulienetti, R., Kalter, H. and Davis, N.C. (1962). Amniotic fluid volume and experimentally-induced congenital malformations. Biol. Neonat. 4, 300-309.

Halitsky, V. and Krumholtz, B.A. (1970). Amniotic fluid analysis in erythroblastosis fetalis. Am. J. Obstet. Gynec. 106, 1209-1221.

Hamerton, J.L., Doran, T.A. and Swyer, P. (1974). Canadian guidelines for antenatal diagnosis of genetic disease: a joint statement. C.M.A. Journal 3, 180-181.

Hanid, T.K. (1975). Pneumothorax and surgical emphysema in a newborn baby caused by amniocentesis. Br. J. Obstet. Gynec. 82, 170-171.

Harris, J.S.W. (1964). Oligohydramnios and cortisone-induced cleft palate. Nature 203, 533-534.

Harrison, R.G. and Malpas, P. (1953). The volume of human amniotic fluid. J. Obstet. Gynec. Brit. Emp. 60, 632-639.

Hertig, A.T. (1945). On the development of the amnion and exocoelomic membrane in the pre-villous human ovum. Yale J. Biol. Med. 18, 107-115.

Hertig, A.T. (1960). Pathological aspects. In, the Placenta and Fetal Membranes (ed. Villee, C.A.). Williams and Wilkins Co., U.S.A. pp. 109-124.

Hoyes, A.D. (1968). Electron microscopy of surface layer (periderm) of the human foetal skin. J. Anat. 105, 149-161.

Huisjes, H.J. (1968). Cytologic features of liquor amnii. Acta cyto. (Balt.) 12, 42-45.

Huisjes, H.J. (1970). Origin of the cells in the liquor amnii. Am. J. Obstet. Gynec. 106, 1222-1228.

Huisjes, H.J. (1973). Cytology of the amniotic fluid and its clinical applications. In, Amniotic Fluid: Research and Clinical Application (eds. Fairweather, D.V.I. and Eskes, T.K.A.B.). Exerpta Medica, Amsterdam, pp. 95-132.

Hutchinson, D.L., Gray, M.J., Plentl, A.A., Alvarez, H., Caldeyro-Barcia, R., Kaplan, B. and Lind, J. (1959). The role of the fetus in the water exchange of the amniotic fluid of normal and hydramniotic patients. J. Clin. Invest. 38, 971-980.

Ivy, R.H. (1957). Congenital anomalies. Plast. reconstr. Surg. 20, 400-411.

Jeffcoate, T.N.A. and Scott, J.S. (1959). Polyhydramnios and oligohydramnios. Can. Med. Assoc. J. 80, 77-86.

Josimovich, J.B. (1973). Hormones in the amniotic fluid. In, Amniotic Fluid: Research and Clinical Application (eds. Fairweather, D.V.I. and Eskes, T.K.A.B.). Exerpta Medica, Amsterdam, pp. 212-222.

Kalter, H. (1968). How should times during pregnancy be called in teratology? (editorial). Teratology 1, 231-234.

Kendrick, F.J. and Feild, L.E. (1967). Congenital anomalies induced in normal and adrenalectomized rats by amniocentesis. Anat. Rec. 159, 353-356.

Kino, Y. (1972). Reduction malformations of the limbs in the rat fetus following amniocentesis. Cong. Anom. 12 (1), 35-44.

Kohler, H.G. (1962). Congenital transverse defects of limbs and digits (intra-uterine amputation). Arch. Dis. Child. 37, 263-275.

Krag, D. (1974). Amnionic rupture and birth defects of the extremities. Hum. Pathol. 5, 69-77.

Landing, B.H. (1950). Amnion nodosum: A lesion of the placenta apparently associated with deficient secretion of fetal urine. Am. J. Obstet. Gynec. 60, 1339-1342.

Leist, K.H. and Grauwiler, J. (1974). Fetal pathology in rats following uterine-vessel clamping on day 14 of gestation. Teratology 10, 55-68.

Lind, T. (1973). The biochemistry of the amniotic fluid. In, Amniotic Fluid: Research and Clinical Application (eds. Fairweather, D.V.I. and Eskes, T.K.A.B.). Exerpta Medica, Amsterdam, pp. 60-81.

Lind, T. and Billewicz, W.Z. (1971). A point-scoring system for estimating gestational age from examination of amniotic fluid. Brit. J. Hosp. Med. 5, 681-685.

Lind, T. and Hytten, F.E. (1970). Relation of amniotic fluid volume to fetal weight in the first half of pregnancy. Lancet 1, 1447-8.

Love, A.M. and Vickers, T.H. (1972). Amniocentesis dysmelia in rats. Br. J. exp. Path. 53, 435-444.

Love, A.M. and Vickers, T.H. (1973). Vasopressin-induced dysmelia in rats and its relation to amniocentesis dysmelia. Br. J. exp. Path. 54, 291-297.

Mahmood, K. and Altshuler, G. (1975). Amniotic band syndrome in an immature fetus. Obstet. and Gynec. 45, 349-351.

Makepeace, A.W., Fremont-Smith, F., Dailey, M.E. and Carroll, M.P. (1931). The nature of the amniotic fluid. Surg. Gynec. Obstet. 53, 635-644.

Makowski, E.L., Prem, K.A. and Kaiser, I.H. (1956). Detection of sexes of fetus by the incidence of sex chromatin in nuclei of cells in amniotic fluid. Science 123, 542-543.

McIntosh, R., Merritt, K.K., Richards, M.R., Samuels, M.H., and Bellows, M.T. (1954). The incidence of congenital malformations: A study of 5,964 pregnancies. Pediatrics 14, 505-521.

McInroy, R.A. and Kelsey, H.A. (1954). Chorioangioma associated with acute hydramnios. J. Path. Bact. 68, 519-523.

Menees, T.O., Miller, J.D. and Holly, L.E. (1930). Amniography. Preliminary report. Amer. J. Roentgenol. 24, 363-366.

Menkes, B., Sandor, S. and Ilies, A. (1970). Cell death in teratogenesis. In, Advances in Teratology 4 (ed. Woollam, D.M.H.). Logos Press, London, pp. 169-211.

Moore, K.L. (1973). The Developing Human: Clinically Oriented Embryology. W.B. Saunders Co., Philadelphia, p. 97.

Moore, K.L., Graham, M.A. and Barr, M.L. (1953). The detection of chromosomal sex in hermaphrodites from a skin biopsy. Surg. Gyn. Obst. 96, 641-648.

Mossman, H.W. and Noer, H.R. (1947). A study of the amnion with the electron microscope. Anat. Rec. 97, 253-257.

Nadler, H. (1970). Risks in amniocentesis. In, Early Diagnosis of Human Genetic Defects (ed. Harris, M.). U.S. Government Printing Office, Washington, D.C., pp. 129-148.

Nadler, H.L. and Burton, B.K. (1973). Enzymes in the amniotic fluid and the pre-natal diagnosis of inborn errors of metabolism. In, Amniotic Fluid: Research and Clinical Application (eds. Fairweather, D.V.I. and Eskes, T.K.A.B.) Exerpta Medica, Amsterdam, pp. 223-261.

Nadler, H.L. and Gerbie, A. (1971). The present status of amniocentesis in intra-uterine diagnosis of genetic defects. Obstet. Gynec. (N.Y.) 38, 789-799.

Nicholas, J.S. (1962). Experimental methods and rat embryos. In, The Rat in Laboratory Investigation, 2nd Ed. (eds. Farris, E.J. and Griffith, J.Q.). Hafner Publishing Co., New York, pp. 51-67.

Ornoy, A., Sekeles, E. and Sadovsky, E. (1974). Amniogenic bands as a cause of syndactyly in a young human fetus. Teratology 9, 129-133.

Parmley, T.H. and Seeds, A.E. (1970). Fetal skin permeability to isotopic water in early pregnancy. Am. J. Obstet. Gynec. 108, 128-131.

Patten, B.M. (1957). Varying developmental mechanisms in teratology. Pediatrics 19, 734-748.

Patterson, T.J.S. (1959). Congenital deformities of the hand. Ann. roy. Coll. Surg. Engl. 25, 306-330.

Patterson, T.J.S. (1961). Congenital ring-constrictions. Brit. J. plast. Surg. 14, 1-31.

Patterson, T.J.S. (1962). Amniotic bands. In, The Human Amnion and Chorion (Bourne, G.L.). Yearbook Medical Publishers, Inc., Chicago, pp. 251-264.

Persaud, T.V.N. (1972). Effect of intra-amniotic administration of hypoglycin B on foetal development in the rat. Exp. Path. 6, 55-58.

Persaud, T.V.N. (1973). Meromelia and other developmental abnormalities in experimental oligohydramnios. Anat. Anz. Bd. 133, S. 499-502.

Plentl, A.A. (1961). Transfer of water across the perfused umbilical cord. Proc. Soc. exp. Biol. & Med. (N.Y.) 107, 622-626.

Plentl, A.A. (1966). Intra-uterine operations on Rhesus monkeys. Trans. New Eng. Obstet. Gynec. Soc. 18, 75-90, 1964.

Plentl, A.A. and Gray, M.J. (1954). Hydrodynamic model of a 3-compartment catenary system with exchanging end compartments. Proc. Soc. exp. Biol. (N.Y.) 87, 595-600.

Plentl, A.A. and Gray, M.J. (1957). Physiology of the amniotic fluid and the management of hydramnios. Surg. Clin. N. Amer. April, 405-419.

Plentl, A.A. and Hutchinson, D.L. (1953). Determination of deuterium exchange rates between maternal circulation and amniotic fluid. Proc. Soc. exp. Biol. (N.Y.) 82, 681-684.

Poswillo, D. (1972). Experimental first-trimester amniocentesis in non-human primates. Teratology 6, 227-234.

Poswillo, D.E. and Sopher, D. (1971). Malformation and deformation of the animal embryo. Teratology 4, 498.

Queenan, J.T. (1973). Amniotic fluid proteins, amniotic fluid amino acids and their clinical significance. In, Amniotic Fluid: Research and Clinical Application (eds. Fairweather, D.V.I. and Eskes, T.K.A.B.). Exerpta Medica, Amsterdam, pp. 194-211.

Räihä, C.N.R. (1963). Organic acids in fetal blood and amniotic fluid. Pediatrics 32, 1025-1032.

Sachs, L., Serr, D.M. and Danon, M. (1956). Prenatal diagnosis of sex using cells from amniotic fluid. Science 123, 548.

Saunders, P. and Rhodes, P. (1973). The origin and circulation of the amniotic fluid. In, Amniotic Fluid: Research and Clinical Application. (eds. Fairweather, D.V.I. and Eskes, T.K.A.B.). Exerpta Medica, Amsterdam, pp. 1-18.

Schreiner, W.E. (1967). The composition of amniotic fluid in its relation to fetal health. In, Intra-Uterine Dangers to the Foetus. (eds. Horsky, J. and Stembera, Z.K.). Exerpta Medica, Amsterdam, p. 379.

Schweichel, J.-U. and Merker, H.J. (1973). The morphology of various types of cell death in pre-natal tissues. Teratology 7, 253-256.

Scott, J.S. and Bain, A.D. (1958). Amnion nodosum. Proc. roy. Soc. Med. 51, 512-513.

Seeds, A.E., Jr. (1968). Amniotic fluid and fetal water metabolism. In, Intra-Uterine Development (ed. Barnes, A.C.). Lea and Febiger, Philadelphia, pp. 129-144.

Seeds, A.E. and Hellegers, A.E. (1968). Acid-base determinations in human amniotic fluid throughout pregnancy. Am. J. Obstet. Gynec. 101, 257-260.

Shaw, R.E. and Marriott, H.J. (1949). The origin of amniotic fluid and the bearing on this problem of fetal urethral atresia. J. Obstet. Gynaec. Brit. Emp. 56, 1004-1009.

Singh, S., Mathur, M.M. and Singh, G. (1974). Congenital anomalies in rat foetuses induced by amniocentesis. Ind. J. Med. Res. 62, 394-401.

Singh, S. and Singh, G. (1973). Hemorrhages in the limbs of fetal rats after amniocentesis and their role in limb malformations. Teratology 8, 11-18.

Singh, S. and Singh, G. (1974). The role of uterine contraction in producing hemorrhages in rat foetuses after amniocentesis. Teratology 10, 145-147.

Streeter, G.L. (1930). Focal deficiencies in fetal tissues and their relation to intra-uterine amputations. Contr. Embryol. Carneg. Instn. 22, 1-44.

Taussig, F.J. (1927). The amniotic fluid and its quantitative variability. Am. J. Obstet. Gynec. 14, 505-517.

Torpin, R. (1965). Amniochorionic mesoblastic fibrous strings and amnionic bands: Associated constricting fetal malformations or fetal death. Am. J. Obstet. Gynec. 91, 65-75.

Torpin, R. (1968). Fetal Malformations Caused by Amnion Rupture During Gestation. Charles C. Thomas Pub., Springfield, Illinois, pp. 129-148.

Trasler, D.G, Walker, B.E. and Fraser, F.C. (1956). Congenital malformations produced by amniotic sac puncture. Science 124, 439.

United Nations Scientific Committee on the Effects of Atomic Radiation (1958). General Assembly Official Records 13th Session, suppl. 17 (A/3838) p. 228.

Vosburgh, G.J., Flexnor, L.B., Cowie, D.B., Hellman, L.M., Proctor, N.K., and Wilde, W.S. (1948). The rate of renewal in women of the water and sodium of the amniotic fluid as determined by tracer techniques. Am. J. Obstet. Gynec. 56, 1156-1159.

Votta, R.A., Bobrow de Gagneten, C., Parada, O. and Gulietti, M. (1968). Cytologic study of amniotic fluid in pregnancy. Am. J. Obstet. Gynec. 102, 571-577.

Wachtel, E., Gordon, H. and Olsen, E. (1969). Cytology of amniotic fluid. J. Obstet. Gynaec. Brit. Cwth. 76, 596-602.

Wislocki, G.B. and Dempsey, E.W. (1955). Electron microscopy of the human placenta. Anat. Rec. 123, 133-149.