A CHROMOSOMAL ANALYSIS OF 25-DAY PIG EMBRYOS

by

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INTRODUCTION

It is a well established fact that prenatal death losses in swine greatly exceed the death losses from parturition to marketing. This fact points out one of the most perplexing problems facing the swine industry today; i.e., pig losses before farrowing.

When the reproductive capabilities of swine are compared with cattle, sheep or horses, it is clear that swine have a much greater reproductive potential because they are a polytocous species with a shorter generation interval. However, these higher ovulation rates are followed by an increase in the percentage of embryos that die *in utero*. The estimated losses range from 30 to 50% with approximately 75% of this wastage occurring early in gestation.

It has been estimated that a sow must produce 5 to 6 pigs at term to pay for production costs. Studies have shown that the average number of ova shed is 15 while the average number of pigs farrowed per litter is only 9.4. Another 2.2 pigs are lost before marketing. These prenatal deaths represent not only a tremendous loss in dollars invested in the breeding program but also dollars lost from the potential profit from the lost pigs. This emphasizes the necessity for a better understanding of the factors and mechanisms causing embryonic losses in swine.

The purpose of this study was to investigate the possible relationship between chromosomal anomalies and early embryonic death.

LITERATURE REVIEW

General Reproductive Processes in Swine

Fertilization rates in swine have been estimated from 95 to 98% (Robertson et al., 1951; Self et al., 1955; Haines et al., 1959; Perry and Rowlands, 1962). According to Hancock (1961), penetration of the ova by the sperm and pronuclei formation does not occur earlier than 6 hours after mating. The time of mating has an influence on the time of first cleavage. Hancock (1961) estimated that first cleavage takes place no earlier than 21 hours after mating in sows mated normally. He suggested that the second cleavage takes place approximately 12 hours later. However, cleaved ova were recovered earlier than 21 hours from sows following delayed matings.

Fertilized ova enter the uterus in the 4-cell stage from 60 to 84 hours after the onset of estrus (Pomeroy, 1955). Blastocysts were first seen on the 6th day and all fertilized eggs had reached this stage before the 8th day (Perry and Rowlands, 1962). Corner (1923) and Perry and Rowlands (1962) concluded that intra-uterine migration and spacing of the embryos take place before the blastocysts elongate. Perry and Rowlands (1962) observed a 300-fold increase in the length of the blastocyst at 16 days compared to a 9-day old blastocyst, which was followed by another slight distension before the establishment of the blood-vascular system.

Perry and Rowlands (1962) observed that the uterus increases in length throughout the first 18 days following mating; however, the elongation was most rapid between the second and sixth days and represented a 50% increase in length. The length of the uterus was not found to be

related to the number of corpora lutea or the number of surviving embryos at any stage examined but was correlated with the increase in weight of uterine tissues. The average length of the uterine horns on the third day and on the 13th and 18th day was 190 cm and 360 cm, respectively.

Scope and Causes of Prenatal Mortality

Prenatal development of mammals can be divided into three periods: the ovum, the embryo and the fetus. Hanly (1961) stated that embryonic death, as used by most authors, describes losses occurring primarily in the first two periods and, to a lesser extent, in the early part of the fetal period. However, Healy (1961) stated that prenatal losses can best be described as occurring either before or after implantation.

Hanly (1961) reviewed the studies on prenatal mortality in farm animals and reported mortality values ranging from 23 to 50% in sows and gilts, 20% in heifers and cows without histories of infertility and 20 to 30% in sheep. In cows with histories of infertility, prenatal mortality was 59%. The major portion of the loss occurred prior to the 25th day of gestation in swine. Comparable losses in laboratory rats have been reported by Asdell (1969) at about one-third, Harper (1964) at 19.5%, McLaren and Michie (1959) at 18.7% and Battaglia (1969) at 20.8%.

Since Hammond (1914) first called attention to the incidence of embryonic mortality in pigs, much research effort has been directed toward the elucidation of the factors associated with prenatal mortality. Corner (1923) classified the causes of embryonic death into the following categories: defects of zygosis, defects of the zygote and defects of the maternal environment. Hafez (1968) listed heredity, nutrition, age of dam, overcrowding *in utero*, hormonal imbalances and thermal stress as factors contributing to prenatal mortality.

Although the amount of prenatal losses has been examined extensively, the precise time of embryonic death and the factors and mechanisms operating to govern these losses remain relatively obscure. Runner (1951) attributed embryonic death losses to the inherent limitations within the individual embryo or to the maternal limitations which affect the litter. Later, Hafez (1968) stated that embryonic losses may be due partially to the fetal-maternal interactions.

Hanly (1961) postulated several causative agents affecting prenatal mortality. He listed infections, aging of gametes, inbreeding and genetic abnormalities, hormonal imbalances, nutritional factors, age and parity of dam and isoimmunization to be most important. Boyd (1956) stated that two main factors involved in embryonic death in cattle, sheep and swine are genetics and environment. Under the genetic factors he included breed, family, inbreeding, blood groups and lethal and semi-lethal factors. Under environmental factors he included nutrition, age, season, condition at service, exogenous hormones and infections as being most important.

Many factors have been shown to increase or decrease prenatal death losses, but no factor or combination of factors has eliminated these death losses in a group of animals (Hanly, 1961). It was further stated that this residual embryonic mortality seemed to be a relatively constant entity in nature and could be explained only by a more universally active factor.

Other Factors Associated with Prenatal Mortality

Intra-uterine Crowding of Embryos: Most accounts of prenatal mortality in swine assume that the fault rests in the uterine contribution to

infertility. It has been shown that increases in ovulation rates are accompanied by higher uterine mortality. Rathnasabapathy et al. (1956) proposed that intra-uterine crowding of the embryos may be a possible cause of the reduction in litter size. Hancock and Hovell (1962) and Hunter et al. (1967) improved the technique of egg transfer in the sow and greatly enhanced the study of this possibility in embryonic losses.

Gibson et al. (1963), Longenecker et al. (1965) and Wood et al. (1967) noted that superovulated gilts were subject to elevated rates of embryonic losses but, at birth, litter sizes were not significantly greater in superovulated gilts than in the controls. Attempts to increase litter size in laboratory animals, either by hormonal superovulation (Fowler and Edwards, 1957; Adams, 1960; Hafez, 1964) or by superinduction of embryos (McLaren and Michie, 1959; Adams, 1960), have been unsuccessful and in some instances litter size was reduced.

Hafez (1964), working with rabbits, concluded that as the number of implantation sites increase, the vascular supply to each particular site was reduced. He postulated that this resulted in the restriction of placental development, with subsequent embryonic death losses. Based on this evidence, workers have postulated that some uterine factor limits the number of embryos which can undergo implantation and placentation. Bazer et al. (1969) called this uterine capacity and defined it as the ability of the uterus to support embryonic development. They tested this postulate by mating boars, which were dominant for white hair color, to cross-bred gilts. Duroc sows were used as donors to provide genetically marked embryos which were homozygous recessive for red hair color. The genetically marked embryos were added to the uterine horns just below

the tubo-uterine junction of the recipients so that the total number of embryos was either 16, 22, or 28. The recipients were slaughtered on the 90th day of gestation. The average litter sizes were found to be 8.8, 8.5, and 9.9 for the groups receiving 16, 22, or 28 embryos, respectively. Litter sizes for the non-operated controls and sham-operated controls were 9.9 and 8.2, respectively, and the values did not differ significantly from the treatment groups. This indicated that the majority of embryonic deaths occurred early in gestation. Therefore, it was hypothesized that the uterus selects those embryos which comprise the litter, since there was no significant difference in survival rates of the native and foreign embryos developing in a common uterus. Increased wastage, associated with increased litter size, appeared to be related to maternal limitations and this uterine factor, which limited litter size, remained at a characteristic level for each species. Therefore, the barrier imposed by uterine capacity must be removed before an improvement of reproductive efficiency in polytocous species can be obtained.

Dziuk (1968) investigated the effect of intra-uterine crowding in gilts by either varying the number of embryos or the length of the uterus. He distributed 130 gilts into the following control and treatment groups: control, restricted, oviduct resection, one horn and ovary removal, superovulated, and egg transfer gilts. Gilts in his study had the ability to maintain 14 embryos for 25 days of gestation. Intra-uterine crowding was a limiting factor in embryonic survival only when this number was exceeded. He concluded that overcrowding <u>in utero</u> was unlikely a factor affecting prenatal losses early in gestation. Yet, Bazer (1969) concluded

that the primary mechanism responsible for controlling litter size occurs prior to the 25th day of pregnancy.

Age of dam: There is a positive correlation between augmented age in sows and reduced reproductive efficiency (Boyd, 1965). Many investigations have revealed increased ovulation rates with advancing age in sows. Perry (1954) noted higher corpus luteum numbers with age beyond the fifth litter, with the end result being increased embryonic losses. Both Perry (1956) and King and Young (1957) agreed that the incidence of embryonic mortality was greater in older sows. Biggers et al. (1962) demonstrated the progressive diminution of litter size in mice with age in terms of litter order and Lipschutz et al. (1963) showed similar results in mice in terms of maternal age.

Biggers (1969) reviewed the problem of early embryonic death losses with emphasis on the estimation of the uterine contribution to infertility which operates on the embryo. It was suggested that certain congenital abnormalities in polytocous species declines with maternal age, since competition between individuals in utero increases with maternal age. The general problem at the uterine level is to determine what part of the infertility is due to an increase in abnormal embryos and what part is due to uterine failure. Two causes of uterine failure were apparent: a generalized aging process and a cumulative local effect depending on the number of fetuses. The final conclusion was that the evidence points to uterine aging as the limiting factor of reduced fertility.

Level of Nutrition: The influence of level of nutrition on embryonic death loss has received considerable attention in the pig. Several studies have indicated that increased energy intake in gilts results in

higher ovulation rates and increased embryonic mortality (Robertson et al., 1951; Christian and Nofziger, 1952; Self et al., 1955; Haines et al., 1959).

Haines et al. (1959) reported on a two year study of the effect of two levels of energy intake on reproductive phenomena in gilts. One group was full-fed and the other group had the energy intake reduced to 50% of the full-fed group. They reported embryonic losses of 22.1% and 11.6% at 25 days of gestation for the full-fed and restricted groups, respectively. Embryonic losses from the period of 25 days to parturition were reported as 9.7% and 8.5% for the two groups, respectively. Their findings indicated that restricting energy intake delayed puberty, depressed ovulation rates and decreased embryonic mortality. They concluded that gilts should be full-fed for high ovulation rates but limited-fed to attain higher survival rates.

Bazer et al. (1968) presented a study which attempted to explain the increased rate of embryonic death associated with short term ad libitum feeding and embryo superinduction in gilts. They assigned at random 190 gilts to two basal feeding levels of either 1.81 kg (L) or 2.72 kg (H) per day. One-half of the gilts in each group were flushed 14 days prior to breeding and were then returned to their normal feeding level. Complete embryo transfers were performed between the low (L) and low flushed (LF) groups, and between the high (H) and high flushed (HF) groups, and the average number of embryos was increased to 17.5, 19.7, 21.5 and 22.5 in the L, LF, H, and HF groups, respectively. However, the average litter size at birth was not significantly different among treatments or control groups. Embryos from flushed and non-flushed gilts were equally

viable and the reduced rate of embryonic survival in gilts with high energy intake prior to breeding must be caused by some uterine factor which effectively limits litter size. This limit, which was defined as uterine capacity, is not greater than what is now reflected in normal litter size in gilts.

Battaglia (1969) studied the effect of feeding chlorpropamide, a hypoglycemic agent, for the first 25 to 30 days of gestation on various reproductive phenomena in gilts and sows. Chlorpropamide feeding resulted in an increased number of embryos and a decreased preimplantation loss in gilts and sows when measured at 25 days of gestation. In gilts, he found an increase in the number of young farrowed but showed no increase or even a slight decrease in birth weight, in number of pigs weaned, in weaning weights, and in postnatal losses. Chlorpropamide feeding was shown to exhibit the reverse effects in sows; the number born was greater in the control sows but the number of postnatal losses were decreased in the treatment group. No effect was reflected in birth and weaning weights. The glycogen content of the fetuses of the treated sows was higher at 25 days of gestation but lower at 45 days.

Effect of Hormones: An imbalance of progesterone and estrogen has been hypothesized as a causative agent of embryonic mortality. However, the use of hormonal therapy to increase embryonic survival rates has been both inconclusive and inconsistent.

Pomeroy (1955) stated that the rapid passage of ova into the uterus may be associated with larger amounts of progesterone being produced by multiple ovulations. However, Spies et al. (1959) failed to note any difference in the rate of passage of eggs into the uterus in progesterone

treated and control gilts. Loy et al. (1958) observed a lack of association between the number of corpora lutea and the total progesterone content of the luteal tissue. Their findings failed to support the idea of multiple ovulations producing excess progesterone with subsequent increased embryonic mortality.

Haines et al. (1958) found no influence on embryonic survival rates from administering 25 mg of progesterone in sows every other day for the first 25 days of gestation. Also, Sammeliwitz et al. (1956) gave daily dosages of 50 to 100 mg of progesterone and obtained decreasing rates of embryonic survival. Nevertheless, Day et al. (1959) combined progesterone and estrogen in a ratio of 2000:1 and obtained a decrease in embryonic mortality of approximately 11% in the treated group.

Most authors tend to agree that the administration of exogenous hormones probably exhibits its most profound effect in reducing prenatal mortality in animals which have a deficiency of endogenous gonadal hormones (Boyd, 1965).

Genetical Aspects of Prenatal Mortality: Probably the least studied factor associated with prenatal mortality is the genetic relationship. However, there is sufficient evidence to suggest that the genetic contribution to prenatal mortality may be considerable in a species such as the pig (Biggers, 1969). Yet, most reports assume the genetic contribution to prenatal mortality to be low. For example, Hanly (1961) assumed that since the heritability estimates for infertility are low, the heritability estimates of prenatal death are low, also. Nevertheless, Bishop (1964) theorized that an "unsuspectedly" large part of embryonic death in cattle could be attributed to genetic causes. These losses were classified as

reproductive wastage and the theory was advanced that early embryonic losses are unavoidable and should be regarded as a means of eliminating unfit genotypes in each generation at a low biological cost. It was inferred that many of the genetic factors are not inherited but arise de novo in each parent generation.

Boyd (1965) noted a range in phenotypic expression of genetic influences on female fertility, such as the production of abnormal ova, the absence of gonadal tissue and the production of ova which could be fertilized and give rise to defective zygotes. Knudsen (1956) demonstrated that chromosomes with inverted segments could arise during the first stage of the reduction division in spermiogenesis. During pairing and crossing-over between the inverted and a normal chromosome, a loose fragment may be formed. These sperm appear normal and are capable of fertilizing an ovum but an unbalanced genetic complement will result which may cause the death of the embryo.

Boyd (1965) reported that different breeds of pigs show a wide range of fertility, as measured by litter size. Several authors have observed breed differences in prenatal mortality (Reddy et al., 1958; Robertson et al., 1951a), while others have shown that no breed differences exist (Robertson et al., 1951b; Self et al., 1955; Baker et al., 1958). However, most differences have been demonstrated to be due in part to variation in ovulation rates. There have been many observations in pigs with emphasis on families and lines, which showed differences in ovulation rates and embryonic survival rates. Perry (1960) proposed that the sire could have a significant effect on the repeatability of embryonic losses in the successive litters of his daughters.

Positive correlations between inbreeding and increased prenatal death have been shown in several experiments. Sang (1956) stated that for each 20% increase in inbreeding there is a reduction of one pig in litter size. King and Young (1957) found that litter size in influenced by the inbreeding of the dam rather than the inbreeding of the embryo. The effect of inbreeding in the dam appeared to operate through a depression of ovulation rate rather than through embryonic death.

Cytogenetics and Congenital Disease

Of increasing importance during the past decade has been the advancement of the field of cytogenetics. Although chromosome abnormalities have been known to exist in both plant and animal species for a very long time, most of the earlier work was centered around assessing specific chromosome numbers characteristic of various species. However, recent progress in the area of medical cytogenetics, relating specific types of chromosomal abnormalities to various syndromes in man, has prompted research in the area of chromosomal aberrations associated with spontaneous abortions in humans.

Swanson, Merz and Young (1967) discussed four classes of chromosomal aberrations leading to the rearrangement of the linear order of genes. They are deletions or deficiencies, duplications, inversions and translocations. The first three classes affect only a single chromosome; whereas, the latter involves more than one chromosome. These structural aberrations result from chromosomal breakage and reunion in various patterns differing from the normal sequence of the loci. The cause of spontaneous chromosomal breakage is unknown. Ring chromosomes and isochromosomes were also mentioned briefly as structural modifications.

Heller (1969) discussed two types of abnormalities involving chromosome number. Firstly, euploidy deals with a balanced chromosome set in which the somatic complements are exact multiples of the basic number for the species. These multiples, which occur as variations in the number of chromosomes from the normal paternal and maternal complements, are labeled polyploidy. Secondly, he described aneuploidy as irregular numbers of chromosomes involving individual chromosomes within a set. Bartalos (1967) stated that polyploidy could arise when nuclear division occurs without simultaneous divisions of the cytoplasm. Nondisjunction and anaphase lagging were considered the most frequent causes of aneuploidy.

Chromosome anomalies are relatively frequent events. Heller (1969) calculated chromosomal abnormalities to occur one in 208 newborn infants (0.48%). Carr (1963) estimated that approximately 20% of the spontaneous abortions occurring in the first one-third of human pregnancy are caused by chromosomal aberrations. Of the 12 abortuses he found to be chromosomally abnormal, 3 lacked a sex chromosome, 2 were triploid, 1 was tetraploid, and 5 were trisomics. In a later study (1967) of 227 abortuses, he reported that 22% possessed gross chromosome abnormalities.

The advancement of medical cytogenetics relating various syndromes in man with chromosome aberrations and the work of Carr (1963), correlating spontaneous abortions in humans to chromosome anomalies, quickly enhanced the investigation into chromosomal aberrations of embryos of other mammalian species.

Austin and Braden (1953) delayed the matings in rats from 5 to 9 hours after ovulation and found that 8.8% of the fertilized ova resulted

in dispermy. Other workers have found that dispermy usually results in triploidy. Analysis of the chromosomes of 45 male rats by these workers failed to reveal any triploidy, which supported the hypothesis of Beatty (1951) that triploid embryos rarely survive to birth. Similarly, Edwards (1958a,b) found that heteroploid embryos did not survive to parturition.

Piko and Bomsel-Helmreich (1960) induced polyspermy in rats after treatment with colchicine and slaughtered the rats from 8 to 12 and 13 to 15 days of gestation. They recovered 193 embryos from the 8 to 12 day period and found that 157 were diploid, 19 were triploid, 19 were mosaic and 5 were regressing. From an analysis of 111 embryos at 13 to 15 days of gestation, they found 98 normal, 3 mosaic and 10 regressing. They concluded that: (1) dispermic fertilization results in triploidy in the rat, (2) most triploid embryos can become implanted, (3) a large portion of the heteroploids die soon after implantation, and (4) the reduction of the mosaics after 12 days gestation implies that the chances of these forms reaching birth are extremely remote.

Bomsel-Helmreich (1961) delayed the matings in sows 44 to 78 hours after the onset of estrus and slaughtered them at 18 and 26 days following mating. Chromosomal analysis showed that in the 13 sows slaughtered at 18 days gestation, 6% of the embryos were either triploid or mosaic, whereas no triploid or mosaic embryos were found in the 14 sows slaughtered 26 days after mating.

Shaver and Carr (1967) studied chromosomal abnormalities associated with delayed fertilization in New Zealand and Flemish Giant breeds of rabbits. Intravenous injections of 25 i.u. of chorionic gonadotrophin were administered to insure ovulation within 10 hours. The does were

mated at intervals from 0 to 14 hours after injection. They recovered 154 blastocysts from 23 does on the 6th day. Chromosomal analysis revealed 13 abnormalities (12%) in the 135 blastocysts suitable for karyotyping. Triploidy was the most common anomaly and was found in six instances in rabbits which had been mated 8 to 9 hours after injection. They also found 1 pentaploid, 2 mixoploids and 1 trisomy among the embryos from rabbits that were mated immediately after injection.

McFeely (1967) slaughtered 7 gilts 10 days after mating and found that 9 of the blastocysts (10%) possessed grossly detectable chromosomal anomalies of which 4 were triploid, 3 tetraploid, 1 diploid/triploid, and 1 deletion. In addition, 2.3% of the blastocysts were apparently degenerate. He hypothesized that approximately one-third of prenatal mortality is caused by grossly detectable chromosome abnormalities.

Hofsaess (1969) found chromosome aberrations in 6 of 46 rabbit embryos recovered from California does and in 3 of 29 rabbit embryos recovered from Dutch-Belted does, or a total of 12% abnormal embryos. No significant difference was noted between breeds.

OBJECTIVES

(1) To determine what chromosomal abnormalities, if any, are present in the 25-day pig embryo.

(2) To compare the karyotypes prepared from leucocyte cells from the dam with the karyotypes prepared from her 25-day embryos.

MATERIALS AND METHODS

Experimental Animals

Nine phenotypically normal purebred Hampshire and crossbred Hampshire x Yorkshire gilts were mated to two purebred Hampshire boars. The gilts used in this study were obtained from the V.P.I. swine herd. They were maintained in pasture lots and were fed ad libitum on a 15% fortified corn-soybean ration.

Estrus was checked twice daily with the aid of a boar and each gilt was mated on the first day of heat and again on the second day if she would accept the boar. All gilts were sacrificed on the 25th day following the first mating by severing the juglar vein and the reproductive tracts were removed within five minutes.

Blood samples were drawn from five of the gilts and one of the boars for leucocyte cultures.

Blood Culture Procedure

Ten ml of blood were collected in a sterile 15 ml centrifuge tube containing 0.2 ml of heparin[1]. A rubber stopper was inserted immediately and the tube was gently inverted a couple of times for mixing. The original stopper was discarded, the blood was allowed to drain from the mouth of the tube, and another stopper was inserted. The tube was allowed to stand at room temperature from 2 to 4 hours until adequate sedimentation of the red blood cells had taken place. Three ml of the plasma were transferred to a culture bottle containing 7 ml of TC-Medium 199[2] plus 100 to 200 USP units of penicillin-G[3], 100 to 200 mcg of

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1 Sodium heparin in a concentration of 1000 units/ml obtained from Organon, Inc., West Orange, New Jersey.

2 Difco Laboratories, Detroit, Michigan.

3 Chas. Pfizer and Co., Inc., New York, New York.

dihydrostreptomycin[4] and 0.04 ml of bacto-phytohemagglutinin-M[5] per ml. The culture was allowed 3 days of undisturbed incubation at 37°C.

Approximately three to five hours before the end of incubation, 0.25 µg of Colcemid[6] was added to each culture bottle to arrest the cells in metaphase. The cells were loosened from the inside of the culture bottle and poured into a 15 ml tube and centrifuged at 130 x g for five minutes. The supernatant was removed and Hanks solution[5] was added in 2 ml portions for five minutes. This was followed by centrifugation at 130 x g.

After the supernatant was removed, a warm (37°C) hypotonic solution (1 part TC 199:4 parts 2X distilled water) was added and the cells were suspended by gentle pipetting and incubated for 15 minutes at 37°C. After five minutes of centrifuging at 130 x g, the supernatant was decanted.

Approximately 4 ml of fixative (1 part glacial acetic acid:3 parts absolute methyl alcohol) were added without disrupting the cell "button". Following 30 minutes of undisturbed fixation, the cells were suspended by gentle pipetting and centrifuged at 75 x g for five minutes. The fixative was removed, fresh fixative was added and the cells were resuspended and allowed to stand for six minutes. The procedure was repeated two or three times until the cells were clean.

The clean cells were resuspended in 1 ml of fresh fixative and affixed to slides by dropping from a small pipette and igniting the alcohol residue on the slide. After the slides were completely dry, they

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4 Chas. Pfizer and Co., Inc., New York, New York.
5 Difco Laboratories, Detroit, Michigan
6 Grand Island Biological Company, Grand Island, New York.

were stained with Giemsa and mounted for scanning. This procedure was a modification of the technique described by Moorehead et al. (1960).

Procedure for Culturing and Processing Embryos

Each gilt was slaughtered on the 25th day after the first mating and the reproductive tract was removed as a unit to avoid contamination. The tract was immediately placed in an insulated water bath at 37°C and transported to the cytogenetics laboratory for processing. Corpora lutea counts were obtained. The mesometrium was trimmed away to allow the uterine horns to be extended and the tract was flamed to avoid contamination. Each uterine horn was bisected at each implantation site and the embryo was removed and placed in the barrel of a 20 cc syringe. The embryo was broken up by forcing it through a 24 gauge needle into a culture bottle containing 10 ml of growth media[7] to which 100 to 200 USP units of penicillin-G[8] and 100 to 200 mcg of dihydrostreptomycin[8] had been added.

All embryos were cultured for three days at 37°C. Three hours prior to termination of the incubation period, 0.25 µg of Colcemid[9] was added to each culture bottle to arrest the cells in metaphase.

At the end of the incubation period, each culture was transferred to a 15 ml centrifuge tube and was centrifuged for five minutes at 300 x g. The supernatant was poured off and 5 ml of a hypotonic solution, consisting of one part fetal calf serum and five parts distilled water, were added. The embryonic material was agitated gently with a pipette

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7 Minimal Eagles medium with 15% fetal calf serum, both obtained from Difco Laboratories, Detroit, Michigan.
8 Chas. Pfizer and Co., Inc., New York, New York.
9 Grand Island Biological Company, Grand Island, New York.

and maintained at 37°C for 12 minutes. It was then centrifuged at 300 x g for 5 minutes and the hypotonic solution was replaced with 5 ml of a fixative containing one part glacial acetic acid to three parts absolute methyl alcohol. Immediately following 15 minutes of undisturbed fixation, the cell "button" was resuspended by gentle pipetting and allowed to fix for an additional 15 minutes. The cells were again centrifuged, the supernatant was replaced with fresh fixative, the cell "button" was resuspended by pipetting and then left undisturbed for a period of 10 minutes. The supernatant was again poured off and the remaining cells were resuspended in one to two ml of fresh fixative. Finally, the cells were affixed to slides, stained with Giemsa and mounted for microscopic examination in the usual manner. Three slides were prepared for each embryo.

Scanning of Slides

All slides were carefully and systematically scanned under a Zeiss microscope using 10X objective and 10X oculars. The location of each usable cell was recorded. Cells to be examined further for photographic reproductions were selected on the basis of apparent integrity of the cell, dispersion of the chromosomes and distinctness of chromosome morphology. Visual counts were made on a minimum of 10 cells per slide, using oil immersion. However, if cells with counts other than the normal diploid number (2n=38) were noted, additional counts were made to eliminate possible errors.

Photographing and Karyotyping for Embryo Analysis

A minimum of two photographs were taken per embryo under oil immersion for karyotype analysis, using a polaroid type 55 positive/

negative film. Negatives were developed and 8 x 10 enlargements were made. From the enlargements, individual chromosomes were cut and aligned on the basis of relative length and centromere position to form the karyotypes. If abnormal cell numbers were found, a minimum of five cells per embryo were karyotyped to establish the abnormality present. Karyotypes from the work of McFee and Banner (1969), and reported in the Atlas of Mammalian Chromosome, were used as a guide in the construction of the karyotypes.

RESULTS

Reproductive Performance

The reproductive performance of the nine experimental gilts is summarized in table I. Ovulation rates ranged from 9 to 15 ova with a mean of 11.77 ± 0.41.

A total of 75 embryos were recovered, of which six (8%) were regressing. Of the six regressing embryos, three were degenerate and had begun to be reabsorbed. An additional three embryos were determined to be regressing since there was an absence of mitotic figures and a preponderance of pyknotic cells was noted.

Based on the number of viable embryos and the total number of corpora lutea, the average amount of embryonic death losses for the nine gilts was 35.9%.

Analysis of Leucocyte Cultures

Table II summarizes the results of leucocyte cultures from five gilts from which blood samples were obtained. From a total of 78 cells counted, 67 (85.9%) of the cells possessed the modal class count of 38 chromosomes. Plate 1 illustrates an example of a normal cell from a leucocyte culture.

The leucocyte culture acquired from the Hampshire boar (#1430) used in this study revealed the normal diploid number of 38 chromosomes in all 12 cells counted. A karyotype from the boar, shown in plate 2, serves as an example of the normal diploid number in the male.

Analysis of 25-Day Embryo Cultures

The distribution of cells with various chromosome numbers from 25-day embryos is summarized in table III. From a total of 797 cell counts,

Table I. Means and Standard Errors for the Reproductive Performance of Nine Gilts Slaughtered at 25-days of Gestation

Gilt number	Gilt weight (kg)	Corpora lutea[a]	Embryos recovered	Embryos regressing	Number viable embryos	Prenatal mortality %[b]
1195	102.97	11	7	--	7	36.4
8-8	112.95	10	8	2	6	40.0
4-8	126.10	12	11	1	10	16.7
4-9	127.91	14	6	2	4	72.4
77-7	138.80	11	9	--	9	18.2
78-4	182.80	13	12	--	12	6.9
49-7	130.18	9	2	--	2	77.8
94-8	151.05	11	11	--	11	---
90-9	156.49	15	9	1	8	46.7
Total	--	106	75	6	69	--
Average	136.58 ± 7.86	11.77 ± 0.41	8.3 ± 1.03	0.7 ± 0.28	7.66 ± 1.19	35.91 ± 9.15

[a] Total number of corpora lutea on both ovaries.
[b] Based on number of viable embryos and total corpora lutea counts.

Table II. Distribution of cells from Leucocyte Cultures of Gilts with Various Chromosome Numbers

Gilt[a]	Chromosome number				
	< 37	37	38	39	> 39
49-7	--	--	8	--	--
77-7	--	3	16	--	--
78-4	--	--	15	--	--
90-9	2	3	13	--	2
94-8	--	1	15	--	--
Total	2	7	67	--	2
% modal class	--	--	85.9	--	--

[a] Leucocyte cultures from 5 gilts obtained from the V.P.I. swine herd.

PLATE 1

KARYOTYPE OF A NORMAL FEMALE
FROM A LEUCOCYTE CULTURE

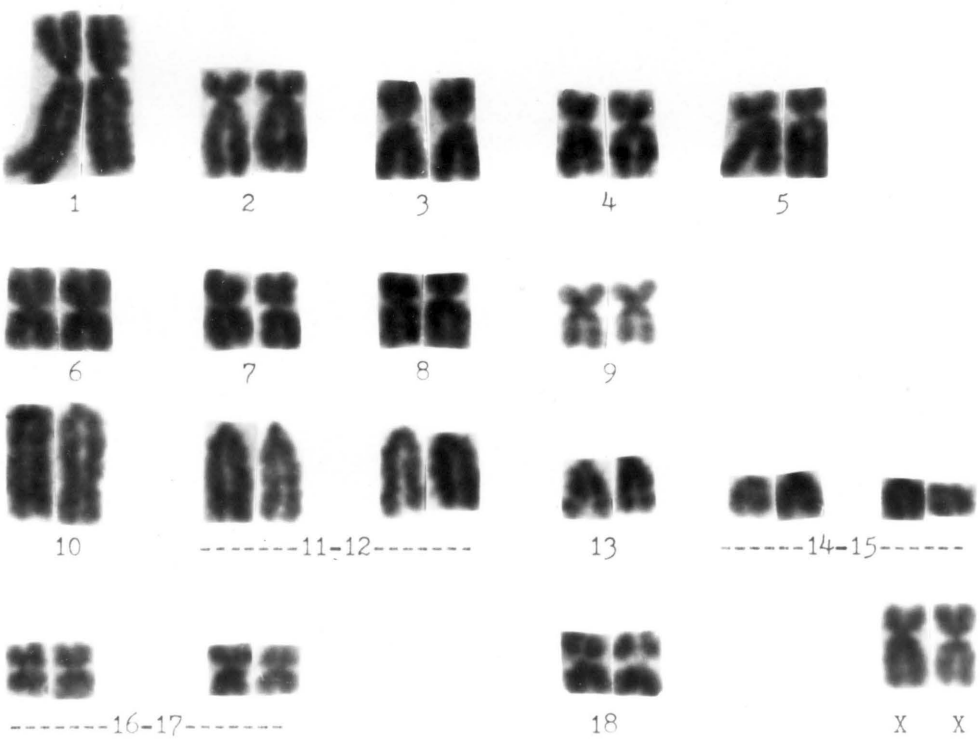


PLATE 2

KARYOTYPE OF A NORMAL MALE
FROM A LEUCOCYTE CULTURE

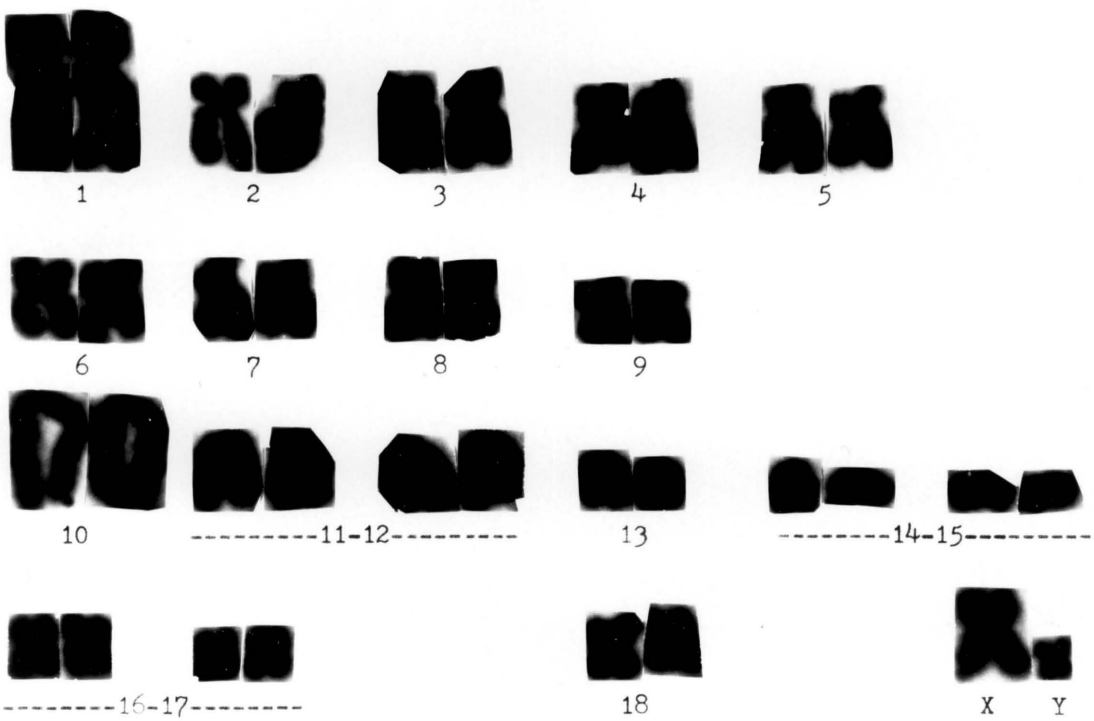


Table III. Distribution of Chromosome Numbers from 25-day Embryo Cells within Litters

Gilt number	Number of embryos	Chromosome number				
		< 37	37	38	39	> 39
1195[a]	6	3	15	86	--	--
8-8	6	--	--	90	--	--
4-8	10	--	2	137	--	--
4-9	4	--	3	60	--	--
77-7	9	8	11	97	--	7
78-4	12	2	--	68	--	1
49-7	2	--	--	9	--	--
94-8	11	2	9	100	--	2
90-9	8	4	9	72	--	--
Total	68	19	49	719	--	10
% modal class		--	--	90.2	--	--

[a] Includes one embryo with 13 counts of 37.

719 (90.2%) were found to contain the expected diploid number of 38 chromosomes. Plates 3 and 4 show the normal karyotypes of a male and female embryo, respectively.

The sex chromosome complex was XY in 57% and XX in 43% of the 67 embryos analyzed. With the exception of one embryo, all embryos possessed the normal complement of 38 chromosomes. Embryo six from gilt 1195 had 13 counts of 38 chromosomes, 13 counts of 37 chromosomes and 2 counts of 36 chromosomes. Karyotype analyses of six cells with counts of 37 chromosomes revealed a monosomic condition of group 16. Karyotype analysis of the two cells with counts of 36 chromosomes showed monosomy of group 16, also. However, the other missing chromosomes were from different groups. Plate 5 shows the karyotyped abnormality of a cell consisting of 37 chromosomes.

PLATE 3

CHROMOSOMES OF A NORMAL MALE EMBRYO

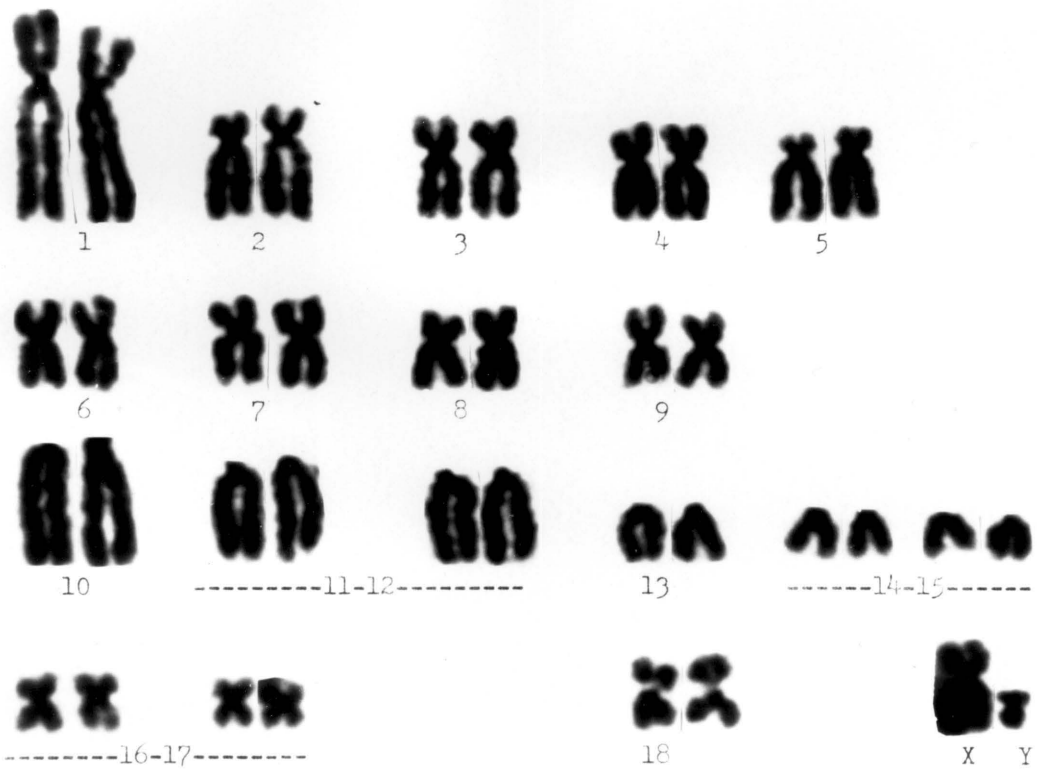


PLATE 4

CHROMOSOMES OF A NORMAL FEMALE EMBRYO

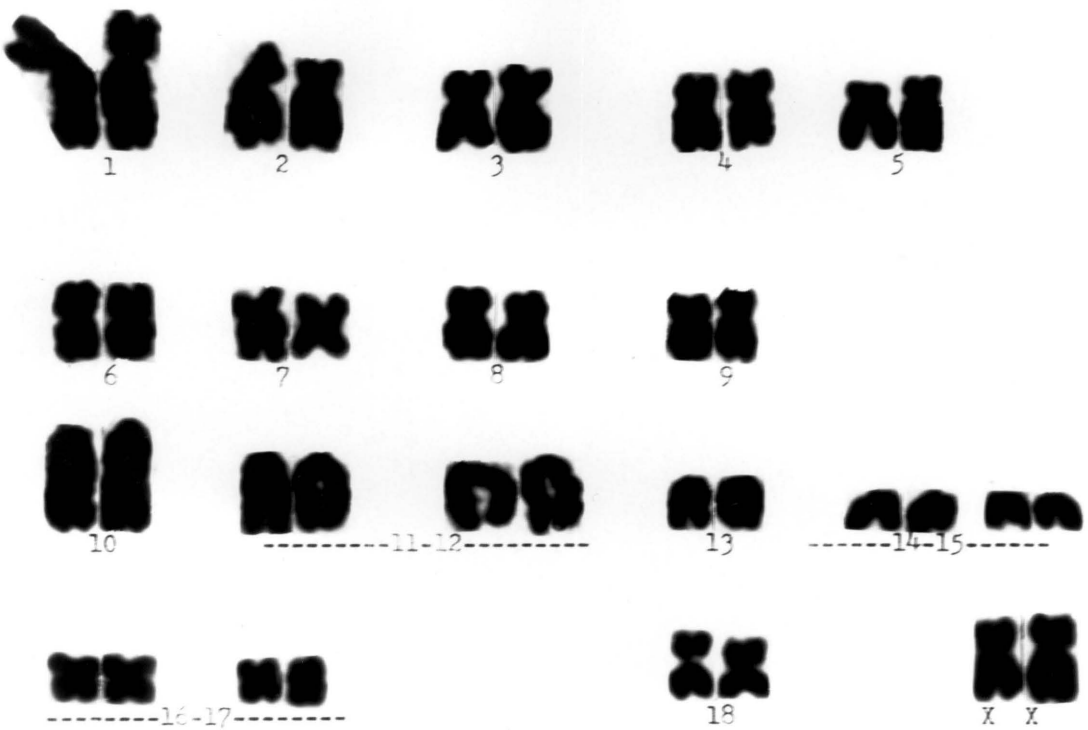
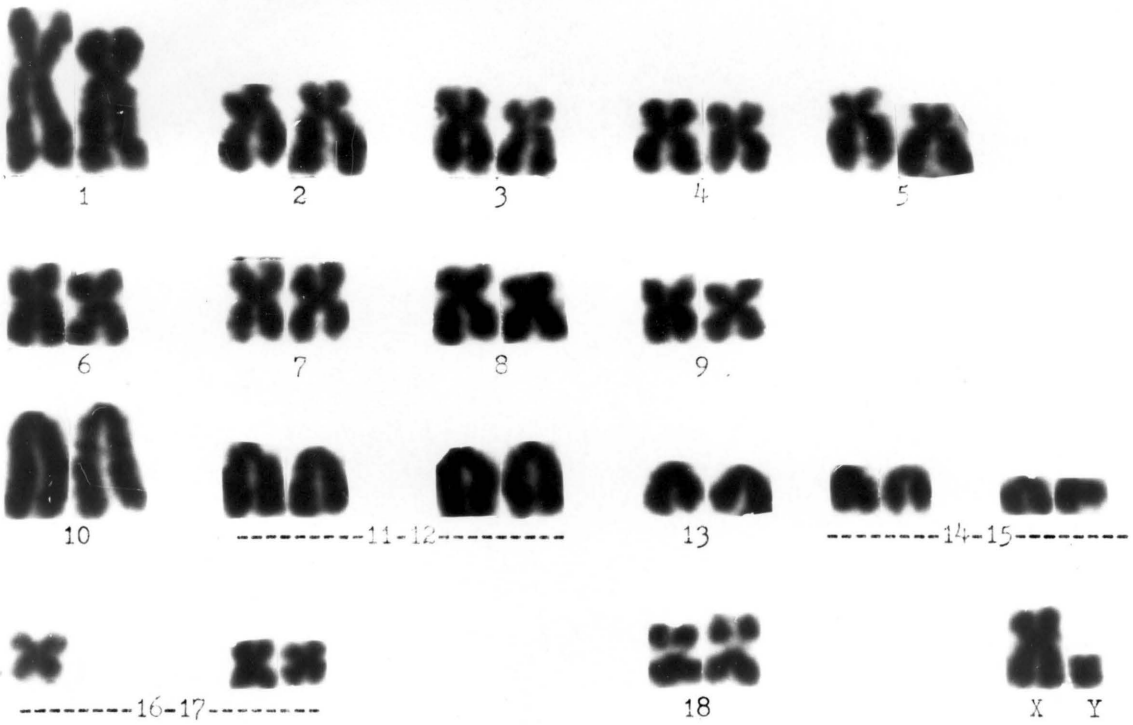


PLATE 5

AN ABNORMAL MALE PIG EMBRYO
MONOSOMIC FOR GROUP 16



DISCUSSION

General Reproductive Phenomena

The mean ovulation rate of 11.77 ± 0.41, with a range of 9 to 15 ova, is comparable to results reported in other studies. McFeely (1966; 1967) reported mean ovulation rates of 10.6 and 12.8, respectively. Battaglia (1969) reported similar results. The number of corpora lutea were counted and used as the criterion for the number of ova shed (Perry and Rowlands, 1962).

The amount of prenatal mortality estimated at 25 days of gestation (35.9%) is within the general range of 33 to 43% noted by Hanly (1961).

Chromosome Number and Morphology

Chromosome number and chromosome morphology are basic to a thorough understanding of the genetics of an organism. Therefore, much of the earlier work in cytogenetics was devoted to assessing chromosome numbers characteristic of various species. Chromosome numbers reported in earlier studies have ranged from 30 to 40 chromosomes. Hance (1917), Makino (1944), Sacks (1954), and Spalding and Berry (1956) found a diploid number of 40 chromosomes in the domestic pig; whereas, counts of 38 were noted by Krallinger (1931), Bryden (1933), Crew and Koller (1939), Muldal (1948), Clausen and Syverton (1961), Ruddle (1961), Gimenez-Martin *et al.* (1962), and Stone (1963).

The modal class number of 38 chromosomes was noted in leucocyte cultures from five gilts in 85.9% of the cells counted in the present study. The normal complement was characterized by 19 pairs of chromosomes; that is, 6 pairs of acrocentrics and 13 pairs having centromere positions varying from median to sub-terminal. These results are in agreement with

Vogt (1968). McConnell et al. (1963) analyzed 152 cells from 14 domestic pigs of six different breeds and found that only 21 cells differed from the normal diploid number of 38 chromosomes.

Sources of Error in Chromosome Numbers

Variability in chromosome number in the leucocyte and embryo cultures can be ascribed mainly to the mechanical limitations of the technique. During the process of suspending the cells in the hypotonic solution and fixative, the cell membrane could be ruptured causing errors in chromosome counts to be encountered. Also, during the process of affixing the cells to slides by igniting the alcohol residue and during the period of flame drying of the slides, the cell membrane could be ruptured causing chromosomes to be lost or extra chromosomes to be counted. With the exception of one embryo, variation in chromosome number was sporadic and no consistent abnormality was present. This suggests that the variation in chromosome number was due to the processing of the embryos. McConnell et al. (1963) found that 21 of 152 cells analyzed differed from the modal class count. Rupturing of the cells, early disjunction of the chromatids and nondisjunction were described as the probable causes of the variability in chromosome counts.

Identification of Sex Chromosomes

The X chromosome has been described as a large metacentric chromosome (McConnell et al., 1963; Hard et al., 1965), as a medium sized chromosome with an almost median centromere (Gimenez-Martin et al., 1962), as a submetacentric chromosome (McFeely and Hare, 1965) and as a subtelocentric chromosome (Stone, 1963).

From the analysis of 25-day embryos, a heteromorphic pair of chromosomes was noted in the material obtained from males and the X chromosome

was a submetacentric chromosome. McConnell et al. (1963) reported an XY sex complex since a heteromorphic pair of chromosomes was found.

Cornefert-Jensen et al. (1968) found that the X chromosomes of the pig are submetacentric but are not readily discernible from some of the other submetacentric chromosomes. However, they noted that one of the submetacentric chromosomes continues to replicate DNA later than the others in female metaphases but not in male metaphases. This "late-labelling" chromosome was identified as one of the X chromosomes. It was also shown that the X chromosome and one other submetacentric pair can usually be identified by their centromere indices alone; whereas, the remaining submetacentric chromosomes can be identified by their centromere indices and total length.

Chromosomal Analysis of the Embryo Cultures

During the past 10 to 15 years, there has been a tremendous advancement in the techniques employed to study animal cytogenetics. Although much of this information has been confined to humans, it has prompted the investigation into various animal species manifesting a possible disease or abnormality in chromosome complements. Because of the similarities in physiological and anatomical features to those of man, the domestic pig is being used with increased frequency in bio-medical research. Therefore, it seems reasonable to assume that the investigation of cytogenetics in swine may prove beneficial not only in terms of improvement and production but also to the total understanding of mammalian genetics.

The finding of 57% males and 43% females is in general agreement with the work of Cox (1960). He found that swine have an excess number

of males at birth with a subsequent higher mortality during early life. Shaver and Carr (1969) found that the sex chromosome complex of 6-day rabbit blastocysts were XY in 54% and XX in 46% of the blastocysts.

Since genetic defects affect each embryo individually, cytogenetical studies enable one to determine the contribution of chromosomally defective embryos to prenatal mortality. The finding of no polyploid and only one monosomic embryo in this study is in partial agreement with Bomsel-Helmreich (1961). He reported that 6% of the pig embryos at 18 days of gestation were triploid; whereas, no abnormalities were present at 26 days of gestation. It could be postulated, then, that the mosaic embryo found in this study could have arisen from anaphase lagging or nondisjunction.

It is becoming increasingly apparent that chromosomal abnormalities occur in embryos of mammalian species and the ultimate effect on the viability of the affected blastocyst is becoming established (Austin and Braden, 1953; Beatty, 1957; Bomsel-Helmreich, 1961; Carr, 1963; Bomsel-Helmreich, 1965; McFeely, 1967; Shaver and Carr, 1967; Hofsaess, 1969; Shaver and Carr, 1969). It has also been shown that delayed fertilization may cause polyploidy by inducing the suppression of the second polar body, polyspermy, or fragmentation of the nucleus and blastomeres. Triploidy has been established as the most common abnormality but the precise timing of embryonic death and the factors governing cellular death with the various types of chromosomal aberrations remains unclear.

From the findings of 6% triploid embryos at 18 days of gestation, reported by Bomsel-Helmreich (1961), and since no triploid embryos were

noted in this study or in the 26-embryo study by Bomsel-Helmreich (1961), it could be postulated that triploid embryos can rarely survive implantation. Also, lethal chromosome combinations cannot be excluded as a cause of cell death in the six (8%) regressing embryos found in this study.

The fact that no tetraploid or mosaic embryos were found at 18 or 26 days of gestation by Bomsel-Helmreich (1961) and that only one mosaic embryo was found in this study encourages one to hypothesize that these embryos die in the earlier stages of implantation. Approximately one-half of the cells analyzed from the monosomic embryo were normal. The large number of normal cells might account for its survival of implantation. However, it is unlikely that this embryo would have survived until parturition. Similar studies have shown that chromosomally defective fetuses have rarely survived to birth (Braden and Austin, 1954; Edwards, 1958a,b; Piko and Bomsel-Helmreich, 1960; Bomsel-Helmreich, 1961; Bomsel-Helmreich, 1965; Austin, 1967). In addition to the regressing embryos noted, several sites of complete embryo reabsorption were found on the reproductive tract of the experimental subject.

Bomsel-Helmreich (1965) found that the development of abnormal rabbit blastocysts approximates that of a normal one. Heteroploid mortality apparently was not the result of an impossibility in establishing a normal fetal-maternal circulation but death occurred at some stage of embryonic organization independent of implantation. The postulate was advanced that heteroploid lethality resulted not because of mechanical disturbances or chromosomal activity but probably because of an action on cellular metabolism at the time of differentiation.

Hofsaess (1969) postulated that the abnormal constitution of a developing rabbit embryo could exhibit its ultimate effect in two different ways. Firstly, since an abnormality arising during oogenesis could affect some or many of the templates necessary for cellular division, death of the embryo may result before implantation. Secondly, abnormal embryos which develop from normal gametes at or after fertilization would likely survive through the blastula stage. However, after gastrulation begins and new proteins are needed for cell differentiation then death would occur as a result of the abnormal constitution of the embryo.

From the present study, as well as from other studies, the fate of the abnormal pig embryo and the direct causes for cellular death are not clearly understood. However, it is apparent that the triploid pig embryo can rarely survive implantation. The death of the tetraploid and mosaic embryos occurs during the earlier stages of implantation because of an action on cellular metabolism at the time of differentiation. It is also becoming apparent that the fate of some of the abnormal blastocysts is expressed shortly after fertilization. Therefore, more research in this area is needed to find the direct causative agents in the death of the developing abnormal embryo.

SUMMARY

Nine phenotypically normal purebred Hampshire and crossbred Hampshire x Yorkshire gilts were slaughtered on the 25th day after first mating and the embryos were processed for chromosomal analysis. Blood samples were drawn from five gilts and one boar for leucocyte cultures and a comparison was made between karyotypes prepared from the dam and those prepared from her 25-day embryos.

The mean ovulation rate for the nine experimental gilts was 11.77 ± 0.41. A total of 75 embryos were recovered and six of these embryos were determined to be regressing. Prenatal death losses were calculated to be 35.9%.

Leucocyte cultures prepared from five gilts revealed a modal class number of 38 chromosomes in 85.9% of the cells counted. All cells counted from the leucocyte culture of the boar showed a normal complement of 38 chromosomes.

The expected diploid number of 38 chromosomes was found in 90.2% of the cells counted from the embryo cultures. Karyotype analysis showed only one abnormal embryo, which was monosomic for group 16. Sources of error or variation in chromosome number were described as the mechanical limitations of the technique used. During the process of suspending the cells in the hypotonic solution and fixative, the cell membrane could be ruptured and cause chromosomes to be lost or extra chromosomes to be counted.

It was concluded that in swine the chromosomally abnormal embryos are unable to survive implantation. However, the large number of normal cells present in the monosomic embryo might account for its survival of

implantation. It was postulated that the embryo would not likely survive to parturition, probably because of an adverse effect on cellular metabolism due to the abnormal constitution of the embryo.

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A CHROMOSOMAL ANALYSIS OF 25-DAY PIG EMBRYOS

by

James H. Smith

Abstract

Nine phenotypically normal purebred Hampshire and crossbred Hampshire x Yorkshire gilts were mated to purebred Hampshire boars to produce 25-day embryos for chromosomal analysis. Blood samples were drawn from five of these gilts and one of the boars for leucocyte cultures and a comparison was made between karyotypes prepared from the dam with those prepared from her 25-day embryos.

The mean ovulation rate of the nine gilts was 11.8 ± 0.4. A total of 75 embryos were recovered, of which six were degenerating. In addition to the regressing embryos, several sites of complete embryo reabsorption were found. Prenatal losses were calculated to be 35.9%.

The diploid number of 38 chromosomes were found in 85.9% and 90.2% of the cells counted in the leucocyte and embryo cultures, respectively. A normal chromosomal complement was displayed in all cells examined from the leucocyte culture obtained from the boar.

Karyotype analyses showed only one abnormal embryo, which was monosomic for group 16. Other variations in chromosome number were described as the mechanical limitations of the technique used. If chromosomal abnormalities are constant in nature, these results would indicate that most abnormal embryos are unable to survive implantation, which is in agreement with findings of other researchers. Although the monosomic embryo had survived implantation, it is highly unlikely that it would have survived through parturition.