March 1998

Manual on Pig Embryo Transfer Procedures

Japan Livestock Technology Association
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この印刷物は、JRA 日本中央競馬会の特別振興資金による助成事業（畜産技術協力体制強化「技術移転用教材整備」事業）により、（社）畜産技術協会の委託に基づき農林水産省家畜改良センターのご協力を得て、（社）国際農業交流基金が作成しました。
Preface

The Manual on Pig Embryo Transfer Procedures was prepared by the Japanese Society for Development of Swine New Technology. This Society was established by the National Livestock Breeding Center, which is under the Ministry of Agriculture, Forestry and Fisheries.

The Japanese Society for Development of Swine New Technology was established as a means of improving the mastery of pig embryo transfer techniques by personnel at animal experimental farms run by Japan's various prefectural governments.

This manual was compiled in order to help technical personnel improve their knowledge of the basic technology in this field and stay abreast of the latest breakthroughs in a number of areas, including non-invasive transfer techniques and cryopreservation of pig embryos.

Porcine embryo transfer techniques (together with transgenesis technology) constitute the fundamental basis for organ xenotransplantation and it is universally agreed that these techniques will come to be used extensively throughout the world.

Nevertheless, we have yet to find a pig embryo transfer manual published anywhere in the world that emphasizes the techniques used in embryo transfer.

Thus, with an eye to helping technicians and researchers throughout the world who are working to master porcine embryo transfer techniques, we decided to have this manual (which has already been published in Japanese) translated into English and published.

We sincerely hope that this English manual will prove to be a valuable reference for those engaged in international technology cooperation projects.
A Note from the Editors

Remarkable progress has been made in embryo transfer techniques, particularly with regard to cattle. The first successful pig embryo transfer was reported in 1951, the same year as for cattle. In the case of cattle, this marked the beginning of research activity aimed at developing practical applications for these techniques in developed countries. With pigs, however, research and development of practical applications has proceeded at a slower pace. Pigs produce multiple embryos and have a shorter gestation period, for which reason it was thought that embryo transfers could not be expected to yield economic benefits. In recent years, however, the scientific community has achieved a much better understanding of the physiological characteristics of porcine reproduction, while great advances have been made in genetic engineering and other related fields. As a result, it is thought that embryo transfer technology can be applied to the development of new techniques for manipulation of pig embryos.

However, those of us actually involved in pig embryo transfer research have not achieved the same degree of technical progress that has been seen with cattle embryo transfer work, and it appears that all of us are unsure what approach is likely to yield positive results. There are several reasons for this situation. The swine’s reproductive system is more anatomically complex than that of the cow, and many points regarding reproductive physiology are still not understood. In addition, it is a simple matter to insert the arm into the rectum of a cow, but the smaller size of the pig makes this quite difficult. To solve these difficulties, it is necessary that the National Livestock Breeding Center establish a means for the exchange of information regarding new pig embryo transfer techniques, and that the Center engage in joint efforts to develop practical methods of pig embryo transfer. To meet these needs, the Japanese Society for Development of Swine New Technology (the membership of which comes from industry, government, and academia) was established in 1992. This Society organizes conferences that bring together under one roof workers engaged in pig embryo transfer research for the private sector, prefectural governments, and universities. In the following year, in order to establish a division of responsibilities, the Japanese Society for Development of Swine New Technology agreed to establish committees on transfer and preservation techniques. This manual has been prepared by the Japanese Society for Development of Swine New Technology and its Editorial Committee with an eye to including the views of all researchers and technicians who are engaged in efforts to develop pig embryo transfer techniques. For this reason, our policy has been to write the manual in such a manner that any reader would easily understand it and be able to proceed directly with embryo transfer procedures. This means that there are many places in this manual where we discuss embryo transfer procedures which have not yet gained complete acceptance. In such cases, we have presented descriptions of different methods so that the reader can decide for himself which is best. In particular, there is much room for high pregnancy rate but are plagued by poor implantation rates. To aid future efforts to improve transfer techniques, we have included descriptions of these transfer techniques in an appendix at the back of this manual. We have also included an appendix with the latest information on cryopreservation techniques for pig embryos. Japanese technical terms in this field have not been unified. Usage in the original Japanese text follows that found in “Shin Hanshokugaku Jiten” [New Dictionary of Breeding Terms], published by Buneido. Finally, animals have been treated with care based on a rigorous animal welfare policy.

Japanese Society for Development of Swine New Technology
Manual on Pig Embryo Transfer Procedures, Editorial Committee
Commentary

It was a pleasure to be asked to review the English translation of this Japanese Manual on Pig Embryo Transfer Procedures. Of particular interest, it has been an opportunity to work once again with Dr. Eiji Fuku (ALVIS Inc.) who spent several productive years with me in Canada when he was a graduate student.

As the editors have noted, successful porcine embryo transfer was first reported in 1951, and considerable research was carried out in the early 1960's with the technique. The surgical procedures used today do not differ greatly from those developed at that time, although I believe that this is the first comprehensive hands-on manual to describe techniques used in the pig.

The collection and transfer of porcine embryos have always had application in research, from the early studies on the uterine migration of embryos to such issues as maternal recognition of pregnancy, maintenance of pregnancy, and uterine capacity and litter size. More recently, reciprocal transfer of embryos of different breeds has been used in efforts to identify the breed carrying genes responsible for improved embryonic survival and carcass traits. To date, practical application of embryo transfer techniques has been almost limited to repopulation associated with disease eradication procedures, although the recent interest in transgenic pigs for xenotransplantation purposes suggests that the demand is likely to increase in future. A few publications that have appeared since this manual was written expand on other newer reproductive technologies now possible in pigs or address improved non-surgical approaches to embryo collection and transfer. They are listed below.


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Manual on Pig Embryo Transfer Procedures

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I. History and Applications

1. History

The impetus for embryo transfer research in the pig, as in the cow, received a big boost in 1951 when researchers in the former Soviet Union reported the first successful embryo transfer\(^1\). By the early 1960s, the use of laparotomies for collection and transfer of embryos had become an established approach\(^2-4\). In 1968, a successful transfer using non-invasive means was reported\(^5\) and, in 1970, pig embryos were transported intercontinentally\(^6\). These successes reflected progress achieved previously with bovine embryos. The first pig embryo transfer in Japan was reported in 1973\(^7\). Thereafter, a series of breakthroughs in the development of cryopreservation techniques was reported by a number of researchers, primarily in Japan. With regard to non-invasive techniques for the collection, and especially transfer, of pig embryos, it was only very recently that major advancements have been realized in a number of different countries. Successful attempts at non-invasive embryo collection have been reported from pigs with surgically shortened uteri, but many problems remain unresolved, so this cannot yet be considered a fully developed technique. Table I-1

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1951</td>
<td>First successful pig embryo transfer</td>
<td>Kvasnicki(^8)</td>
</tr>
<tr>
<td>1968</td>
<td>Pregnancies through non-invasive embryo transfer</td>
<td>Poige and Day(^9)</td>
</tr>
<tr>
<td>1970</td>
<td>Successful intercontinental transport of pig embryos</td>
<td>Wrathall et al.(^10)</td>
</tr>
<tr>
<td>1973</td>
<td>First reported pig embryo transfer in Japan</td>
<td>Nishikawa et al.(^11)</td>
</tr>
<tr>
<td>1976</td>
<td>Embryo transfer used to establish SPF herd</td>
<td>Cumock et al.(^12)</td>
</tr>
<tr>
<td>1983</td>
<td>Embryo transfers used to repopulate swine herds in connection with eradication of Aujeszky’s disease</td>
<td>James et al.(^13)</td>
</tr>
<tr>
<td>1985</td>
<td>Production of transgenic piglets</td>
<td>Hammer et al.(^14)</td>
</tr>
<tr>
<td>1985</td>
<td>Piglets produced from split embryos</td>
<td>Rorie et al.(^15)</td>
</tr>
<tr>
<td>1986</td>
<td>Piglets produced through in vitro fertilization</td>
<td>Cheng et al.(^16)</td>
</tr>
<tr>
<td>1989</td>
<td>Transcervical embryo collection from sows with surgically shortened uterus</td>
<td>Kobayashi K et al.(^17)</td>
</tr>
<tr>
<td>1990</td>
<td>Piglets produced from embryos frozen at -196°C</td>
<td>Hazeleger et al.(^18)</td>
</tr>
<tr>
<td>1992</td>
<td>Production of chimeric pigs</td>
<td>Oguri et al.(^19)</td>
</tr>
<tr>
<td>1993</td>
<td>Production of piglets through non-invasive embryo transfer [rettrial]</td>
<td>Kamiwazaki et al.(^20)</td>
</tr>
<tr>
<td>1995</td>
<td>Successful freezing of early-stage embryo after delipidization</td>
<td>Reichenbach et al.(^21)</td>
</tr>
<tr>
<td>1995</td>
<td>Direct transfer method used to produce piglets from frozen-thawed embryos</td>
<td>Nagashima et al.(^22)</td>
</tr>
<tr>
<td>1996</td>
<td>Successful storage of embryos through ultrarapid freezing</td>
<td>Kameyama et al.(^23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kobayashi A et al.(^24)</td>
</tr>
</tbody>
</table>
presents a summary of the key achievements to date in the development of porcine embryo transfer techniques.

2. Applications

The potential applications of embryo transfer are different in the case of pigs as opposed to cattle. With cattle, this technique has enabled the establishment of companies in North America to multiply the numbers of genetically valuable dairy animals and of exotic beef breeds of European origin. With pigs, however, the collection and transfer of embryos have been limited primarily to the study of early embryonic development, the establishment of gestation and their relationship to various uterine events. This research has led to a series of discoveries relating to such aspects as embryogenesis from insemination to implantation in the endometrium, embryonic transuterine migration, embryo spacing, embryonic mortality, etc. It is believed that, when fertilization occurs, the sow does not recognize the pregnancy until 13 days after the onset of estrus. During this period, it is impossible under routine breeding conditions to observe, externally, what is happening inside the uterus. Even when fertilization and subsequent embryogenesis proceed smoothly, if the embryo dies by the 12th day, the sow's estrous cycle will continue without interruption, thereby masking an apparent abortion. The use of embryo transfer techniques, however, may assist in narrowing down the possible causes of infertility, and the latter continues to be a major reason for their use. With the successful use of embryo transfer in 1983 to preserve valuable genetic material while eradicating Aujeszky’s disease from swine herds, a practical application for the technique in the swine industry became apparent. In addition, there are a number of potential new applications, thanks to the development of genetic engineering. It may now be possible to use transgenic technology to improve disease resistance, to modify growth characteristics, and to transform the animal into a “bioreactor” for the production of important biopharmaceuticals. Furthermore, there has been a revival of interest in the use pigs (and especially miniature pigs) for medical research. Researchers are working to produce pig models for various types of diseases by introducing human genes, and efforts are underway to establish the pig as a donor of organs for transplantation into human recipients. Pig embryo transfer techniques constitute the basis of these efforts, and for this reason have become the focus of renewed interest.

The commercial benefits of embryo transfers are much greater in the dairy and beef industries than the swine industry for a variety of reasons. The main reason is the fact that sows bear multiple litters and have a relatively short gestation period, which reduces the value of induced superovulation and multiple births. Secondly, the range of applications is narrowed by the fact that laparotomies performed under general anesthesia are required for efficient collection and transfer of embryos. A third constraint
is the fact that cryopreservation techniques for porcine embryos are not as advanced as those for bovine embryos. Despite these limitations, many of the applications of cow embryo transfer may also be viable in the case of pigs. At present, the following commercial applications of pig embryo transfer techniques appear to be feasible.

(1) Expansion of SPF (specific pathogen-free) herds

In general, it is possible to transmit genetic information to the next generation without the risk of vertical transmission of infectious organisms. To achieve this, early-stage embryos to be transferred are collected from the reproductive tract before contact between the fetal and maternal placentae is established. The embryos must be collected while they are still enclosed by the zona pellucida, so that any pathogens adhering to the zona pellucida can be removed by following recommended washing and dilution procedures. A primary SPF pig cannot be produced through the use of embryo collection and transfer alone (with natural birth, passage through the birth canal and intake of colostrum make it impossible to produce the conditions necessary for SPF), but increased production of primary SPF pigs from super sows can be achieved by combining embryo transfer with a prior step, in which primary SPF pigs are acquired by either hysterectomy or cesarean section. It is then possible to increase production of secondary SPF pigs without spreading disease.

(2) Eradicating disease (such as Aujeszky’s) from a swine herd

Once disease breaks out in a breeding herd, the farm must stop marketing breeding stock. Through the use of embryo transfer, however, embryos can be collected from the infected herd and transferred to a noninfected herd, thereby effectively repopulating while preserving the genetic characteristics of the infected herd.

(3) Taking maximum advantage of superior breeding sows

There may be times when a genetically superior breeding sow is unable to carry a pregnancy to term due to age or poor health. By collecting embryos from such a donor sow and transferring them to a recipient, it may be possible to continue obtaining offspring from this superior animal.

(4) Making efficient use of prepubertal gilts (shortening the generation interval)

For gilts produced from genetically superior sows, hormone treatments can be used to induce estrus in the gilts before they have actually reached puberty. By collecting and transferring the embryos from these gilts, offspring can be obtained earlier than is possible through traditional reproductive management schemes.

(5) Creation of new swine herds (introduction of new genetic stock)

For closed swine herds with a high inbreeding index, embryo transfers can be used to introduce new genetic stock without exposing the herd to disease.

(6) Acceleration of herd improvement through the maternal line

By transferring embryos from a genetically superior sow to produce sires for artificial insemination, herd improvement can be accelerated.
(7) Rapid increase in production of specific breeds

By transferring embryos of a breed that is in high demand to recipients of another breed, rapid production of animals of that specific breed can be facilitated.

(8) Economic transport of breeding animals

Embryo transfer can be used to facilitate inexpensive transport of breeding males or females. In future, when embryo preservation and sexing techniques become practical, pig embryo transfer operations will become even more efficient and attractive.

With regard to development of non-invasive embryo transfer and cryopreservation techniques, major improvements have occurred only recently, so the range of applications for pig embryo transfer can be expected to expand greatly in the near future.

Cited references

1. Kvasnickii AV. Interbreed ova transplantation. Sovetsk Zooteh 1, 36-42 (Anim Breed Abstr 1951; 19, 224.).


19. Included in Appendix 2.

20. Included in Appendix 3.

Further suggested readings


I. History and Applications
II. Overview of Embryo Transfer Techniques

The basic techniques involved in pig embryo transfer are shown in the diagram below. More detailed descriptions of each of these techniques are presented in Chapter III.

![Diagram of embryo transfer process]

(Induction of estrus): This term is enclosed in parentheses because induction is unnecessary if estrus occurs naturally.

A roman numeral to the right of an oval indicates the chapter which contains further information on the technique listed inside the oval.

Figure II-1. Overview of pig embryo transfer techniques
II. Overview of Embryo Transfer Techniques

Strictly speaking, the term "embryo transfer technique" refers only to the process that begins with embryo collection and continues through embryo transfer, but in a broader sense this term also refers to the whole range of basic breeding techniques, which includes: monitoring signs of estrus and standing heat; collection and processing of sperm; artificial insemination; confirmation of pregnancy following embryo transfer; etc. As such, in order to develop efficient embryo transfer techniques, it is particularly important to understand the anatomy and physiology of the reproductive tract of the sow.

Reproduction in pigs is very different from other domestic animals. A single litter generally consists of about ten piglets, and the gestation period is relatively short, lasting about 114 days on average. Furthermore, the activity of the gonads is not subject to seasonal influence and variation, thereby making it possible for sows to farrow twice per year. Although laparotomies are required for embryo collection and transfer in pigs, just as with sheep and goats, anatomical differences dictate that different surgical techniques be used. In this chapter, we shall describe the reproductive physiology of the sow, devoting special attention to aspects relevant to embryo transfer.

1. Anatomy and function of the sow's reproductive organs

The sow plays the following roles in reproduction: ① produces eggs; ② supports development of fertilized eggs (embryos) in its uterus; ③ gives birth to fully developed piglets; and ④ produces milk for nourishment of the piglets. The sow thus plays a much more complex role in the reproductive process than the boar, which only produces

![Reproductive organs of the sow and their position](image)

Figure II-1. Reproductive organs of the sow and their position
Source: Swine Science (5th edition) [Reproduced with permission.]
sperm. Accordingly, in order to carry out embryo transfer techniques efficiently, it is extremely important that the practitioner have a good understanding of the anatomy and function of each of the sow’s reproductive organs.

(1) Ovaries

Unlike the testicles, the ovaries are located within the abdominal cavity, caudal to the kidneys, below the crest of the ilium, in front of the pelvis, one each on the left and right sides. The size and shape of the ovaries depend on age and stage of the estrous cycle, and the follicles and corpora lutea are much more prominent than with other domestic animals. The functions of the ovaries are to: (1) produce oocytes; (2) secrete (from the follicles) estrogen, which is a type of steroid hormone; and (3) secrete (from the corpora lutea) progesterone, another type of steroid hormone. The ovaries ovulate at a given frequency, producing 10 to 20 oocytes each time. Oocytes are found within follicles. When follicles reach maturity, they rapidly take on increased fluid and enlarge. As the estrous cycle nears an end, each follicle (ca. 8 mm in diameter) ruptures and releases its oocyte. This process is called ovulation. Immediately following ovulation, a corpus luteum forms at the site of the ruptured follicle. The corpus luteum, as mentioned above, secretes progesterone, which serves several functions. For example, it: (1) plays an important role in preparing the uterus for implantation of the embryo; (2) prevents other oocytes from maturing during gestation; (3) maintains pregnancy; and (4) combines with estrogen and other substances to stimulate development of the mammary glands.

If no oocytes are fertilized, the corpora lutea will regress after a certain period of time has elapsed. New follicles and their oocytes begin to mature, and the estrous cycle resumes. Ovarian cysts, which block ovulation, are thought to be one of the major causes of reproductive failure. A follicle containing an oocyte secretes estrogen, which: (1) stimulates development of female reproductive organs; (2) triggers estrus-related behavior, such as standing heat; (3) stimulates development of the mammary glands; and (4) stimulates sexual characteristics of sows. More than 20 kinds of estrogen have
been identified, including estradiol-17 β, estrone, and estriol.

(2) Oviducts (fallopian tubes)

The oviducts are small tubes which wind through the mesosalpinx and link the ovaries to the uterus. On the ovarian end of the tube, each oviduct broadens out into a funnel-shaped infundibulum which covers most of the ovary. After the ova (eggs) are released, the infundibulum guides them into the oviduct. Within 2 to 3 min after being released, the ova first enter the infundibulum. Once in the infundibulum, the ova are carried to the oviduct, and the movement of the tunica muscularis of the oviduct and the beating of cilia inside the oviduct carry the ova down the oviduct. The joining of sperm and ovum (fertilization) occurs in the upper two-thirds of the oviduct. Fertilized ova (embryos) travel down the oviducts for three to four days before reaching the uterus.

(3) Uterus

The uterus comprises two uterine horns (122-152 cm), one short uterine body (5 cm), and one cervical canal (15 cm). The left and right uterine horns are separated by a septum. The uterine horns of the pig are much longer and convoluted than those of other domestic animals. The cervical canal, which is joined to the uterine body, consists of 8 or 9 cartilaginous rugae to accommodate the spiral-shaped tip of the boar’s penis. Except during estrus and parturition, these rugae are tightly interlocked, and they connect to the vagina. In this aspect the pig differs from the cow, in which the uterus has a clearly apparent external orifice. In natural mating, the boar inserts the tip of its penis to near the second ruga of the cervical canal, where it ejaculates its semen. It is believed that the function of the cervical canal is to prevent invasion of bacteria, thereby preventing infection.

(4) Vagina

The vagina is an elastic tube which serves both as a copulatory organ, and as a passageway for the fetus and placenta during parturition.

Figure II-3. Structure of the uterus

Source: Kachiku Hikaku Kaibou Zusetsu [Illustrated Guide to Comparative Livestock Anatomy] (Yojindo Publishing House, reprinted with permission)
(5) External reproductive organs
The external reproductive organs comprise the vestibule of the vagina, the labia (external part), and clitoris. The labia become swollen and red during estrus, and they relax during birth. In addition, the urethral orifice opens into the vestibule.

2. Internal reproductive secretions of the sow
(1) Sexual maturity of the sow
A sow is considered sexually mature when it is capable of normal estrus, ovulation, fertilization, implantation, pregnancy and parturition. The appropriateness of defining sexual maturity on the basis of body weight or age from birth has come into question, because there is doubt as to whether either of these two factors is an accurate reflection of the level of the sow’s development, i.e.—the maturity of the hypothalamic-pituitary-ovarian axis. Naturally, the speed of a sow’s sexual maturation is also greatly influenced by heredity and nutrition. Most reports suggest that the average sow reaches sexual maturity around 200 to 210 days after birth, but this maturation period varies considerably, ranging from 102 to 350 days, so it may not be very meaningful to discuss this issue in terms of an average sexual maturation rate. In the same manner, weight at sexual maturity varies widely, from 55 to 120 kg. In spite of these great discrepancies, however, age is considered a more accurate indicator of sexual maturity than weight.

The processes of sexual maturation and follicle development are closely related. Both are

As granulosa cells within the follicle increase in number, receptors for FSH and estrogen do likewise. As this process continues, FSH and small amounts of estrogen in the tertile follicles stimulate the LH receptors on the theca interna cells (which secrete androgen and estrogen). FSH- and androgen-sensitive granulosa cells convert androgen to estrogen. The granulosa cells produce follicular inhibin, which feeds back to the anterior pituitary. In addition to inhibin, other non-steroidal substances that control ovarian function include oocyte maturation inhibitors, luteinization inhibitors, luteinization stimulators, and FSH receptor binding inhibitors.

Figure II-4. Follicles and follicular control factors in prepubertal gilts
Source: Christenson RK et al., Control of Pig Reproduction (reproduced with permission)
controlled by the maturation of the hypothalamic-pituitary-ovarian axis. Development of the ovaries during the fetal stage is independent of induction by gonadotropins, but such induction does begin to affect follicle development around 60 days after birth. Between 60 and 100 days after birth, we observe development of the mechanism whereby hormonal secretions from the ovaries produce negative feedback to gonadotropins from the hypothalamus.

When prepubertal gilts approach sexual maturity, the spontaneous pulsatile secretion of luteinizing hormone (LH) occurs with increasing frequency. It is known that when sexual maturity is induced in a sow by exposure to a boar, the concentration of estradiol-17β in the peripheral blood increases. It is not yet known if the increased concentration of estradiol-17β is due to spontaneous LH secretion, but evidence obtained in the study of sheep and mice indicates that LH levels change upon exposure to a mature male.

When a sow reaches sexual maturity, the hypothalamus releases a releasing factor which causes the anterior pituitary to secrete follicle stimulating hormone (FSH). This hormone stimulates development of the follicle, which then secretes small amounts of estrogen. Feedback from the released estrogen causes the hypothalamus to produce a releasing factor which, in turn, triggers the release of LH from the anterior pituitary. This stimulates a brief secretion of progesterone, which combines with the increased level of estrogen that accompanies follicle maturity to bring about the onset of estrus. Estradiol-17β is one type of estrogen present in the blood during estrus. The level of estradiol-17β in the blood is highest on the day before estrus begins. The feedback from this estrogen inhibits the release of FSH-releasing factor from the hypothalamus and, at the same time, stimulates the release of LH-releasing factor. The resulting LH secretion from the anterior pituitary brings the follicle to full maturity, and ovulation occurs. LH levels in the blood of a sow during estrus have been observed to fluctuate greatly. In sows, estrus begins rapidly, and the signs that estrus has begun are very obvious. A large surge of LH occurs at some point between 8 h prior to standing heat and 12 h thereafter, and LH levels are highest between 2 h prior to standing heat and 22 h thereafter. With most sows, the highest LH level generally coincides with the beginning of standing heat. In sexually mature sows, estrus reoccurs approximately every 21 days as long as the sow does not become pregnant. As the cycle progresses, changes occur in the morphology and function of the reproductive organs. These changes, as mentioned previously, are closely related to absolute and relative changes in gonadotropin and sex hormone levels. Recent research suggests that ovarian inhibin and oxytocin secreted by the corpus luteum may play a role in the regulation of sexual functions.

The potency of FSH from the pig's pituitary is second only to that of horses. Commercially sold FSH products are extracted from porcine pituitaries. Accordingly,
pigs are not generally very sensitive to their own abundant hormones, and it is said that they respond very weakly to exogenously administered FSH. Furthermore, if prostaglandin F$_2$α (PGF$_2$α) is administered to a cow during the luteal phase, the corpora lutea regress quickly and estrus begins three to four days after administration, so this technique can be used effectively as a means of controlling the estrous cycle during embryo transfer. In sows, however, administration of PGF$_2$α has little effect until about the 11th day of the estrous cycle, so PGF$_2$α is not very useful for estrous cycle control in this species. Methods of estrous cycle control are discussed in greater detail in Chapters III and IV. From the early stages of a sow's pregnancy, PGF$_2$α causes corpora lutea to regress; thus, PGF$_2$α can be used to induce parturition.

Among multiovulating animals, the pig is an excellent model for studying the control of follicular development. One focus of such studies, for example, is the relationship between different follicles within a single ovary. As follicles complete the process of development, they are replaced by new ones from a pool of reserve follicles within the ovary. This replacement occurs between 14th and 16th day of the estrous cycle. As follicles develop to ovulation, some follicles regress, and at the same time new follicles in the replacement pool are prevented from beginning development. The presence of a dominant follicle has different effects in pigs and cows. In cows, it blocks the development of other follicles, but in pigs it actually stimulates the maturation of non-dominant follicles.  

**2) Inducing estrus in prepubertal gilts**

Until it is first mated and gives birth to piglets, a gilt is unproductive. For this reason, many efforts have been made to hasten sexual maturity through artificial means. Nutritional methods have been attempted, but it appears that the day on which sexual maturity begins is not greatly affected unless the pig is extremely deficient in nutritional factors, such as dry matter intake, energy intake, protein intake, fat intake, or intake of vitamins and minerals. According to one report, "The manner in which each individual pig reaches sexual maturity is genetically determined by the breed, but the speed of normal maturation is affected by the pig's environment." Thus, this report states that the number of days required to reach sexual maturity varies depending on the breed, but there are other reports which state that variation among pigs of the same breed is too great to assess differences between breeds. In particular, these latter reports suggest that environmental factors are very influential. In contrast, it is also reported that sows produced through cross breeding reach sexual maturity more quickly. Opinions differ concerning seasonal effects (hours of sunlight, temperature) upon sexual maturation. It is conjectured that seasonal effects do not constitute a very significant factor in the temperate zones. Many attempts have also been made to ascertain the effect of stress and other social factors upon sexual maturation. Social factors include animals per square meter, transport, shifting between pens, and contact.
with mature boars. Many researchers have reported that contact with mature boars hastens the sexual maturation of prepubertal gilts. In addition, the age of the gilt when it is first brought into contact with boars is very important, with 150 to 170 days described as the optimum time to initiate such contact. Initiating contact earlier than this retards the achievement of sexual maturity. Initiating contact later than this has no effect upon the age at which sexual maturity is attained, which is disadvantageous from an economic standpoint.

It is standard practice in pig embryo transfer programs to administer PMSG (pregnant mare’s serum gonadotropin; also frequently called eCG, which stands for equine chorionic gonadotropin) to prepubertal gilts immediately prior to the onset of puberty in order to induce both estrus and superovulation. This is because gilts are sensitive to exogenous hormones before they reach puberty, while endogenous gonadotropins have relatively little effect during this period. A hormonal approach can also be used to synchronize the estrus of sexually mature sows, because sows respond very well to certain orally administered progestational agents. However, exogenous hormones are not necessarily desirable means of controlling the induction of sexual maturity in gilts. There are many problems associated with the routine administration of extremely high doses of eCG, followed by high doses of hCG (human chorionic gonadotropin) (variable estrus response; reproductive performance is poor compared to cycling animals; maintenance of the corpus luteum throughout gestation is poor; administration upsets the estrous cycle in cycling animals; etc.), but there are few effective alternatives at this time. There is some cause for optimism that simultaneous administration of low dosages of PMSG and hCG could serve as a means of accelerating sexual maturity, but further research is required, especially on the question of how to maintain cyclicity following their use. The little research that has thus far been done on estrogen has yielded conflicting results, so estrogen treatment cannot serve as a routine method. In any case, the response of a gilt that has never farrowed is affected by a large number of factors, so careful scrutiny of all relevant factors is required.

(3) Fertilization

The ideal condition for fertilization is when fertile sperm await the arrival of oocytes in the ampulla of the uterine tube. This is called the fertilization zone. It is reported that pregnancy rates are usually highest when mating takes place between 10 and 25.5 h after the beginning of estrus. In the case of pigs, it takes 3 to 4 h for sperm within the sow’s reproductive system to become fertile, and they generally penetrate the oocyte about 1 to 2 h after initial contact. Unlike with cattle, it is often impossible to estimate the time of ovulation in sows using normal methods of breeding management, so artificial insemination is carried out once every 12 to 24 h while the sow is in standing heat. For this reason, attempts are frequently made to control the timing of ovulation by administering hCG. It is thought that oocytes remain fertile for 10 to 21 h (15 ± 5.5 h),
and that they pass through the fertilization zone a few hours after ovulation takes place. It is believed that sperm, on the other hand, survive for over 40 h within the sow's reproductive organs, and that they remain fertile for 25 to 30 h, which is a relatively long time. After being ejaculated into the cervical canal, sperm migrate through the sow's reproductive system. They are propelled by peristaltic movements of the sow's reproductive organs and by their own movements. The quickest sperm may reach the fertilization zone in less than 30 min, but it takes over 10 h for large numbers of sperm to assemble there. The time period in which successful mating can occur, then, is naturally limited by the factors described above, e.g., timing of ovulation, the period of fertility of the oocytes and sperm, and the timing of the arrival of the sperm in the fertilization zone. Because sows remain in estrus for a long time, ovulation is sometimes delayed. For this reason, it is necessary to carry out insemination multiple times. In any embryo transfer program, it is important to achieve fertilization of every ovulated oocyte to obtain as many embryos as possible, so it is necessary to mate the sow as soon as possible after she commences standing heat. Needless to say, this will enable the production of greater numbers of piglets in regular breeding practice.

(4) Control of follicle development during lactation and after weaning

In most cases, sows do not exhibit signs of estrus for four to six weeks after lactation begins if the piglets are allowed to continue to suckle. Within three to 10 days after weaning, follicle size increases, which leads to ovulation and estrus. It is thus clear that even during lactation, follicles are ready to respond immediately to changes in the level of gonadotropin secretion that is triggered by weaning. However, weaning immediately after parturition often leads to abnormal follicle development (follicular cysts), and prolonged estrus can occur. This is thought to be due to the fact that the mechanism by which LH regulates estradiol production does not function during the first week after parturition. Follicular development during lactation and after weaning can be affected by many factors, including parity, season, nutrition, suckling strength, contact with mature boars, and various hormone treatments. FSH secretion is regulated by non-steroidal ovarian factors such as inhibin, but the secretion of LH during lactation is mainly regulated by the suckling strength of the piglets. Stimulation by suckling clearly limits the rise in the concentration of gonadotropin releasing factor, but once weaning has occurred and the sow is no longer affected by the stimulation of lactation, gonadotropin releasing factor is released in greater concentration from the hypothalamus. This, in turn, raises LH concentration in the anterior pituitary and the peripheral blood, and triggers follicular development.

(5) Endocrine status during gestation and parturition

Once pregnancy is established, the corpora lutea in the estrous cycle do not regress, but instead become corpora lutea of pregnancy and continue to secrete progesterone. In pigs, the corpora lutea are required throughout pregnancy. They reach
maximum size approximately one week after ovulation and remain at that size until the final stage of gestation. Because the corpora lutea are the only source of progesterone for sows, if the corpora lutea are removed at any stage of gestation, abortion will ensue.

In sows, estrogen levels in the blood and urine rise during approximately the first 30 days of gestation. In particular, the level of estrone sulfate, which is thought to come from the fetus, rises transiently to nanogram concentrations. The measurement of estrone sulfate can be used to diagnose the establishment of pregnancy and to estimate the number of fetuses.

Relaxin is produced and stored in the corpora lutea throughout gestation. It is released just prior to parturition, and then disappears when parturition begins. Relaxin dilates the cervical canal, triggers the birth of the fetus, and shortens parturition time.

Oxytocin in the blood increases during birth of the fetus and ejection of the placenta. Oxytocin may not be released until plasma progesterone levels fall below 10 ng/ml.

Prolactin in the blood plasma increases rapidly around one to two days before parturition, then reaches and maintains a still higher level during parturition. On the 5th day after the onset of lactation, the prolactin level drops to a fraction of its peak level. Among the various hormones that control mammary gland function, prolactin plays a central role, and its physiological effects are varied. Its effects upon lactation and ovarian functions in the female of various animal species are well understood. In humans, prolactin decreases sexual activity by inhibiting the release of gonadotropins by the anterior pituitary. This function has not been fully verified in the field of animal breeding, but there is interest in clarifying how prolactin is related to the manner in which reproductive functions are manifested during lactation.

3. General reproductive physiology of the sow

(1) Signs of estrus

In addition to the ovarian changes that occur during ovulation, estrus is accompanied by various internal changes (changes in the uterus, changes in the secretions from reproductive organs, changes in the concentration of sex hormones in the blood, etc.) as well as changes in external signs and altered sexual behavior. Because the onset of estrus comes slowly in sows, the beginning and end of estrus are not readily apparent. However, determining the beginning and end of estrus as accurately as possible is one of the main keys to success in any pig embryo transfer program. For about five days around the time of heat, the sow's external genitalia become red and enlarged, and the vestibule of the vagina becomes red. Externally observable changes usually occur in the following sequence.

a. Early stage of estrus (Proestrus)

The early stage of estrus is defined as beginning when the vestibule of the vagina
and the external genitalia (vulva) start to turn red and enlarge. It continues through the
time that the sow becomes receptive to mating. Reddening and enlargement of the
external genitalia become more apparent each day, and enlargement progresses to the
point where surface wrinkles disappear and the skin of the genitalia takes on a glossy
sheen. As the sow approaches standing heat, a watery, transparent mucus is
discharged from the vulva. The sow becomes restless and, if kept together with other
pigs, she may attempt to mount them. With colored breeds, it is often difficult to
determine whether the external genitalia have become red, in which case it is necessary
to check for reddening of the vestibule of the vagina.

b. Estrus (standing heat)

Estrus (standing heat) is defined as beginning when reddening and enlargement of
the vestibule of the vagina and the external genitalia reach a peak and the sow begins
to stand to be mounted. It continues until the reddening and enlargement wane and the
sow ceases to show standing heat. Estrus lasts an average of 55 h (25-95 h) in gilts,
and an average of 70 h (52-85 h) in sows. If the sow comes into contact with a boar, or
if a person presses on the sow's lower lumbar region, the sow will prick up her ears and
stand still. During this period the sow stops

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>No. of days after standing heat</th>
<th>No. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-cell stage</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2-cell stage</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4-, 8-cell stage</td>
<td>3-4</td>
<td>4-8</td>
</tr>
<tr>
<td>Compact morula</td>
<td>4-5</td>
<td>16-30</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>5-6</td>
<td>60</td>
</tr>
<tr>
<td>Hatching blastocyst</td>
<td>6-7</td>
<td>175</td>
</tr>
<tr>
<td>Hatched blastocyst</td>
<td>7</td>
<td>500</td>
</tr>
<tr>
<td>Hatched blastocyst</td>
<td>9</td>
<td>6000</td>
</tr>
</tbody>
</table>

As these changes occur, the mucous discharge gradually decreases in
volume. Appetite sometimes wanes, and the
sow urinates more frequently. The duration of estrus varies greatly
depending on the

individually, the sow and the

parity, but it generally lasts

one to four days.

Figure II-5. Pig embryo development
Source: David DL, Control of Pig Reproduction II (reproduced with permission)
c. Late stage of estrus (metestru)

The late stage of estrus is defined as beginning at the end of standing heat, and continuing through the time that the sow returns to its normal condition. Reddening and enlargement of the vestibule of the vagina and the external genitalia gradually disappear during this stage. Before collecting the developing embryos, it is very important to first verify that this late stage of estrus has come to an end.

(2) Ovulation

There are different reports regarding the timing of ovulation. Some state that it occurs 27.5 to 36.5 h (31 ± 5.5 h) after the onset of estrus, while others report the corresponding figures to be 38.47 to 42.7 h, and still others report 36 to 50 h. When estrus lasts for two days, ovulation occurs approximately 40 h after the beginning of standing heat. It is believed that if hCG is administered to the sow, ovulation occurs 41 h ± 1 h later. It is reported that ovulation lasts approximately 2 h. The number of ovulations varies widely depending on breed, age, nutrition, heredity, and other factors. It is thought that the oocytes remain fertile for 10 to 21 h (15 ± 5.5 h).

(3) Estrous cycle

The period that runs from the occurrence of one estrus to the next is called the estrous cycle. This period is characterized by the cyclical occurrence of the following ovarian changes: development of follicles, and ovulation; formation of corpora lutea; and regression of corpora lutea. Once the sow has reached sexual maturity, the estrous cycle repeats approximately every 21 days (19 to 23 days; average of 20.4 days for gilts, and 22.2 days for sows) all year round unless pregnancy occurs.

(4) Unique reproductive structures of the pig

Within 2 to 3 h after fertilization, pig oocytes migrate from the ampulla to the isthmic transitional zone of the oviduct, where they remain for approximately 36 h. After that, pig embryos at the 4-cell stage migrate from the oviducts into the uterus at approximately 48 h after ovulation. It is known that the speed at which pig embryos descend the oviducts can be significantly accelerated by the administration of progesterone prior to ovulation. Administration of estrogen has been reported to have the opposite effect, causing pig embryos to remain inside the oviducts until the 21st day. By the 6th day of the estrous cycle, embryos are found near the upper uterine horns before continuing toward the uterus. By the ninth day, some embryos migrate to the opposite uterine horn. After 12 days of this migration, embryos are distributed across the entirety of each uterine horn. On the 12th day, the embryos begin to elongate rapidly, and they emit signals which play a decisive role in prompting recognition of pregnancy. Distribution of embryos throughout the length of the uterine horns occurs by this time, and spacing is completed in preparation for the uterine hypertrophy that accompanies gestation.
II. Overview of Embryo Transfer Techniques

Figure II-1. Calculating the Number of Days since Standing Heat when Using Natural Estrus

<table>
<thead>
<tr>
<th>Heat: H</th>
<th>Days in estrus: D</th>
<th>When standing heat is first detected in the morning</th>
<th>When standing heat is first detected in the afternoon</th>
</tr>
</thead>
<tbody>
<tr>
<td>H &lt; 2D</td>
<td>2D ≤ H &lt; 3D</td>
<td>Day on which standing heat begins = day 1</td>
<td>Day on which standing heat begins = day 0.5</td>
</tr>
<tr>
<td></td>
<td>(Last day of standing heat = day 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3D ≤ H</td>
<td>Day on which standing heat ends = day 1</td>
<td>Day on which standing heat ends = day 0.5</td>
<td></td>
</tr>
</tbody>
</table>

The above descriptions are based on the assumption that estrus checks are performed twice daily, once in the morning and once in late afternoon.

It is believed that the speed of transuterine migration is unaffected by the number of embryos, but is influenced by secretions from the embryos that affect the uterus. Transuterine migration is a very disorderly process, but the number of embryos coming to rest on the uterine horn on the side where they originated is usually greater than the number of embryos that switch to the other side.

In order to sustain gestation, it is said that at least four embryos must survive to the 12th day of gestation. In our view, however, the signal that prompts recognition of pregnancy is not a quantitative signal, but a qualitative one. Once the sow’s pregnancy has been established, the concentration of progesterone is not affected by the number of embryos.

4. Calculating the number of days since standing heat

Sows remain in estrus longer than cows, and the length of time between the onset of estrus and ovulation varies considerably depending on the length of the estrous cycle. For this reason, in order to estimate the time of ovulation it is necessary to accurately gauge the beginning and end of estrus. This is an extremely important point not only when attempting to collect the desired stage of embryos, but also when seeking to synchronize the estrous cycles of embryo donors and recipients.

There is no standard method for calculating embryo age or the number of days since standing heat, so comparison of data can cause errors. Thus, when using natural estrus, we recommend the method put forward in this manual in Figure II-1 for calculating the number of days since standing heat, so that variations in the length of estrus will not cause different estrus observers to produce different estimates of the time of ovulation. When using a prepubertal gilt as the donor, it is more practical to base this calculation on the length of time that has elapsed since the administration of hCG, a method which we have adopted in preparing this manual.
5. Embryo transfer techniques and special characteristics of pig reproductive Physiology

As mentioned above, it appears that at least four embryos must survive within the uterus to the 12th day of gestation if pregnancy is to be maintained in the pig. However, it has been discovered that even when only a single embryo is transferred, the fetus can survive to at least the 100th day of gestation (unpublished data). Because it is unusual for all embryos to survive, however, at least 15 embryos are usually transferred. In the case of pigs, transuterine migration prior to implantation in the endometrium results in roughly equal numbers of embryos becoming implanted in each uterine horn regardless of how many oocytes were released by the left and right ovaries during ovulation. For this reason, it is thought that as long as the timing of an embryo transfer is correct, transferring all embryos to a single oviduct or uterine horn will not create any problems.

Cited reference


Further suggested readings

II. Overview of Embryo Transfer Techniques
III. Preparation of the Embryo Donor

The pig from which embryos are collected is called the donor sow or the embryo donor. In this chapter we shall describe various aspects of the preparation of the embryo donor, including the planning of embryo collection, sow selection, handling of the sow prior to embryo collection, and feeding management of the sow following surgical collection of embryos. In order to ensure that embryos are collected at the right developmental stage to meet the purpose for which they are intended, and to be sure that all necessary genetic traits are obtained under disease-controlled conditions, we shall pay close attention to the timing of collection, the breed of the donor, disease-control measures, the method of estrus induction, the genetic stock of the sire used for mating or artificial insemination, disease-control status for the sire, etc.

<Required equipment and reagents>

<table>
<thead>
<tr>
<th>Product</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>eCG : Equine chorionic gonadotropin (eCG; formerly known as pregnant mare's serum gonadotropin (PMSG))</td>
<td>Induction of estrus in embryo donor</td>
</tr>
<tr>
<td>hCG : Human chorionic gonadotropin (hCG)</td>
<td>Induction of estrus in embryo donor</td>
</tr>
</tbody>
</table>

1. Procedures prior to embryo collection

The procedures to be carried out prior to embryo collection are shown in Figure III-1, and are described in order beginning on the following page.

![Figure III-1. Procedures prior to embryo collection](image-url)
2. Design of an embryo collection plan

When designing an embryo collection plan, it is necessary to consider various factors, including: the stage of embryo development; the method of collection; the breed and quality of the embryo donor(s); disease-control status for the donor(s); the method of estrus induction; the genetic merit of the sire(s) used for mating or artificial insemination; disease-control measures for the sire(s); etc. Table III-1 below lists possible collection times, depending on the use to which the embryos will be put.

<table>
<thead>
<tr>
<th>How embryos will be used</th>
<th>Timing of embryo collection</th>
<th>Flushing sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After HCG administration</td>
<td>After natural estrus</td>
</tr>
<tr>
<td>Production of transgenic pig through microinjection of DNA into pronucleus</td>
<td>48-56 h</td>
<td>1st-2nd day</td>
</tr>
<tr>
<td>Control of disease such as Specific Pathogen Free (SPF) program, etc.</td>
<td>5th-6th day</td>
<td>4-5</td>
</tr>
<tr>
<td>Introduction of new genes into a swine herd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercontinental embryo transport</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use of embryo transfer to eradicate disease from a herd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryopreservation of embryos</td>
<td>7-8</td>
<td>6-7</td>
</tr>
<tr>
<td>Use in experiments</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
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<td></td>
<td>5</td>
<td>4</td>
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<td></td>
<td>6</td>
<td>5</td>
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<tr>
<td></td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>
III. Preparation of the Embryo Donor

3. Points to consider in selecting an embryo donor

When selecting an embryo donor, the following points should be considered. The use to which the embryos will be put is an especially important factor.

1. The donor should satisfy all genetic and disease-control requirements for the use to which the embryos are to be put.
2. The donor must be physically suitable to withstand mating. Its hind legs, in particular, must be strong and healthy.
3. The donor must satisfy all requirements for induction of estrus.
4. The donor must be healthy enough to undergo surgery.

4. Methods of inducing estrus

If one intends to breed donors in natural estrus for embryo collection, it is necessary to raise a very large number of sows, monitor them carefully for estrus, and select animals in which estrous cycles have been accurately determined. This method is, therefore, not very practical. To prepare embryo donors, it is more efficient to induce estrus using one of the methods described below. Which method is most appropriate depends on circumstances at the organization where such operations are carried out. Some researchers have reported simultaneous use of two different types of hormones to induce ovulation; in order to collect as many embryos as possible, eCG is administered to increase the number of ovulations, and hCG is used to induce ovulation at a specific point in time so that embryos can be collected at a targeted stage of development. It is not absolutely necessary to use these hormones, however, and their use is sometimes best avoided, such as when making repeated use of the same donor(s).

There are five main estrus induction methods, listed as items a. through e. in the inset on the following page. The standard eCG dosage is 1,000 IU per head for each of these methods, but the reaction of the ovaries to eCG varies depending on such factors as the type of eCG, its lot number, differences between individual sows, differences between breeds, etc. These factors must be taken into account when purchasing eCG. Especially in large-scale embryo collection operations, it is a good idea to check the type of product and its lot number before using it. In addition, the chance of causing inflammation of the endometrium (endometritis) sometimes increases as the eCG dosage increases. When a sow is relatively unresponsive to eCG, the dosage should be increased to 1,250 IU or 1,500 IU. On the other hand, if administration triggers inflammation of the endometrium (endometritis) or causes increased production of abnormal embryos, the dosage should be reduced to 750 or even 500 IU. As for alternative methods, hCG can be used without eCG, or eCG (400-600 IU) and hCG (200 IU) can be administered together to induce estrus. When administering hormones
or other drugs to a sow, the drug must be injected in the proper part of the body, and
none of it must be spilled. If the sow is kept in a large pen, the person who administers
the injection must corner the sow to insert the needle. Special care is required to ensure
that the sow does not make any sudden movement while the injection is being
administered. If a particular sow has a nervous temperament, it is a good idea to herd it
into a stall to restrain its movement before the injection. In addition, even when estrus is
induced, ovulation may be reduced or prevented altogether if the sow is subjected to
severe stress during estrus. For this reason, care must be taken during this period not
to expose the sow to disease, high heat, or other types of stress.

### Methods of inducing estrus in pigs

- a. Administration of eCG and hCG to a prepubertal gilt.
- b. Administration of either PGF$_2$$\alpha$ or an analogue to a pregnant sow (be-
tween 12th and 40th day of gestation) to induce abortion and make use of
  the subsequent estrus.
- c. Utilization of the estrus which follows weaning.
- d. Oral administration of synthetic progesterone.
- e. Utilization of the natural estrous cycle.

#### a. Administration of eCG and hCG to a prepubertal gilt

This is the most commonly used method. Estrus begins four to five days after
administration of eCG. When selecting a donor from among prepubertal gilts (90-120
kg, five to seven months old), keep in mind the points mentioned earlier. Better results
are obtained when care is taken to select a gilt that is close to puberty (depends on age,
not size).

1. Selection of a prepubertal gilt.
2. Intramuscular or subcutaneous injection of eCG (1,000 IU).
3. Intramuscular or subcutaneous injection of hCG (500 IU) at 72h after
   administration of eCG.
5. Mating or artificial insemination.

#### b. Administration of either PGF$_2$$\alpha$ or an analogue (PGF$_2$$\alpha$-A) to a pregnant sow
(between 12th and 40th day of gestation) to induce abortion to make use of the
subsequent estrus.

This method can be used with pregnant sows until they reach mid-gestation. Either
PGF$_2$$\alpha$ or PGF$_2$$\alpha$-A, which cause corpora lutea to regress, is administered to terminate
pregnancy, and then the estrus which follows is utilized. The first time one of these
substances is administered, estrus generally begins five to six days later. Estrus is successfully induced in a high percentage of the animals, but many other factors must be kept in mind. It is necessary to have a pregnant sow ready to receive the embryos; therefore, it takes a long time to induce an estrus and the costs of space, feed and labor in pig raising operations are considerable. The dosage of PGF$_2$,$\alpha$ and PGF$_2$,$\alpha$-A varies depending on where it is injected. Injection into the external genitalia requires only half the dosage needed for intramuscular injection. In addition, eCG and hCG can be used in combination to induce estrus.

1. Procurement of a pregnant sow (between 12th and 40th day of gestation).
2. First administration of PGF$_2$,$\alpha$ (15.0 mg/head) or PGF$_2$,$\alpha$-A (1.0 mg/head) via intramuscular injection.
3. Second administration of PGF$_2$,$\alpha$ (10.0 mg/head) or PGF$_2$,$\alpha$-A (0.5 mg/head), either 12 or 24 h after the first administration of PGF$_2$,$\alpha$ or PGF$_2$,$\alpha$-A.
4. Administration of eCG (1,000 IU) through intramuscular or subcutaneous injection, 24 h after the first administration of PGF$_2$,$\alpha$ or PGF$_2$,$\alpha$-A.
5. Administration of hCG (500 IU) via intramuscular injection, 72 h after administration of eCG.
7. Mating or artificial insemination.

c. Utilization of the estrus which follows weaning

Five to six days after the normal nursing period (three to five weeks) ends and the piglets have been weaned, the sow usually returns to estrus. This estrus can be utilized. Furthermore, eCG and hCG can be used in combination to induce estrus in these sows. The interval between weaning and the onset of estrus varies, however, depending on such factors as the body condition of the sow, the length of the nursing period, and the parity. It is best to avoid using the following types of sows because good results cannot be obtained: sows that are too fat or too thin; sows for which the nursing period is shorter than three weeks; and sows that have had seven or more parities. Estrus usually lasts longer in sows than in gilts, and ovulation tends to occur later. For this reason, it is necessary to monitor carefully for signs of estrus, so that mating or artificial insemination can be carried out at the proper time. Because ovulation occurs later in embryo donors with a long estrus period, the timing of embryo collection must be adjusted accordingly.
III. Preparation of the Embryo Donor

1. Selection of the nursing sow.
2. Weaning.
3. After weaning, administration of eCG (1,000 IU) via intramuscular or subcutaneous injection.
4. 72 h after administration of eCG, hCG (500 IU) is given via intramuscular injection.
5. Monitoring for estrus.
6. Mating or artificial insemination.

**d. Oral administration of synthetic progestin**

Altenogest (17-α-allyl-estratriene-4-9-11, 17-β-ol-3-one, or allyl-trenbolone, RU-2267, REGU-MATE; Roussel-Uclaf, Hoechst UK Ltd.) is administered orally once per day for a specified period. This substance is a synthetic progestin (progesterone-like), and it extends the luteal phase of the estrous cycle for as long as it is administered. After administration is stopped, estrus takes place and can be utilized. The dose is 20 mg (10-40 mg) per sow per day, with the onset of estrus expected to occur on the fifth or sixth day after the last administration of the hormone. This method can be used with: sows with silent heat; sows from which embryos have been collected; and sows which have just finished nursing. In Japan, however, the sale of this hormone has not been approved, so it can only be used experimentally. Also, this hormone must be administered orally to sows, which must therefore be kept in individual stalls. It also possible when using this method to administer eCG and hCG on either the day of the final administration of synthetic progestin, or the day after, to induce estrus.

1. Selection of mature sows.
2. Transfer to stalls.
3. Oral administration of hormone at a set time each day (hormone sprayed on feed).
4. Administration of hormone for an appropriate duration (18 days in the case of sows with silent heat, and 14 days for sows with clear previous estrous signs).
5. Administration of eCG (1,000 IU) via intramuscular or subcutaneous injection on the day after the final administration of synthetic progestin.
6. Administration of hCG (500 IU) via intramuscular injection 72 h after administration of eCG.
7. Monitoring for estrus.
8. Mating or artificial insemination.
e. Utilization of the natural estrous cycle

The sow's estrous cycle lasts 21 days on average. Administration of eCG on the 15th or 16th day of the estrous cycle (when the beginning of standing heat is taken as day 0) is followed 72 h later by administration of hCG. The onset of estrus is expected on the fourth or fifth day following administration of eCG. It is also possible to utilize natural estrus without administering any hormones at all.

① Selection of sows which have a well-defined estrous cycle.
② Administration of eCG (1,000 IU) on the 15th or 16th day of the estrous cycle via intramuscular or subcutaneous injection.
③ Administration of hCG (500 IU) via intramuscular injection 72 h after administration of eCG.
④ Monitoring for estrus.
⑤ Mating or artificial insemination.

5. Monitoring for signs of estrus

Sows in which estrus has been induced must be checked for signs of estrus at a set time once each morning and late afternoon, and the results must be recorded. Judgment of when standing heat has begun is based on several factors, including the number of days since the administration of eCG, the sow's behavior, the appearance of the external genitalia, and the sow's reaction to pressure on her back. Standing heat can be checked easily and accurately by allowing the sow to come into contact with a boar (standing heat will be clearly observable even if they are separated by a fence). It is recommended that the estrus check sheet be marked as follows: ☺ = standing heat; ○ = shows signs of estrus; △ = shows possible signs of estrus; X = shows no signs of estrus. When monitoring for signs of estrus and standing heat in estrus-induced sows, a number of points should be kept in mind. These are listed below in Table III-2.

<table>
<thead>
<tr>
<th>Signs of estrus</th>
<th>2 days after eCG</th>
<th>Day of hCG administration</th>
<th>1 day after hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>External genitalia</td>
<td>Begin to become reddened and enlarged</td>
<td>Reddened, enlarged</td>
<td>Reddened, enlarged</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mucous discharge</td>
<td>Mucous discharge</td>
</tr>
<tr>
<td>Behavior</td>
<td>Loss of appetite</td>
<td>Mounts other pigs</td>
<td>Stands still, picks up ears (standing heat)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stands still (standing heat)</td>
<td></td>
</tr>
<tr>
<td>Reaction to back pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table III-2. Signs of estrus and standing heat in estrus-induced sows
6. Mating and Artificial Insemination

An estrus-induced sow usually shows signs of standing heat about 24 to 28 h after administration of hCG. When standing heat is detected at the morning estrus check, the first mating or artificial insemination should be carried out that same morning, and the second in the late afternoon of the same day. When standing heat is detected at the late afternoon estrus check, the first mating or artificial insemination should be carried out immediately, and the second on the following morning. If the sow continues to show standing heat during the next estrus check, a third mating or artificial insemination should be carried out. It is essential that all oocytes be fertilized so that the greatest possible number of embryos can be collected; thus, it is important that mating or artificial insemination be carried out several times, and as soon as possible after the sow has commenced standing heat. The boar used for mating should have the genetic characteristics required for the use to which the embryos are to be put, and the required disease-control status must have been satisfied. Furthermore, his reproductive ability must be proven. In principle, a boar can be used for mating once or twice per week, so a certain number of boars must be kept ready. Depending on the use to which the embryos are to be put, it may be advisable to use different boars for the first and subsequent matings when several donors are to be mated at the same time, or to combine mating with artificial insemination. The purpose of such a procedure is to reduce the boar-related factors which might lead to failure to achieve fertilization. The use of artificial insemination can prevent overuse of boars and reduce the number of boars that must be kept in a herd.

7. Preparation for semen

When using embryo transfer to improve a swine herd through propagation of a superior strain of both sire and sow, it is not possible, as in the type of mating program described above, to switch sires from one mating to the next. In order to avoid overusing a sire in such a case, it is necessary to store semen using low-temperature preservation techniques, and to use this frozen semen for at least one insemination of an embryo donor. Much has been written about cryopreservation of semen. One diluent widely used for this purpose is Modena, the components of which are listed in Table III-3. A detailed description of cryopreservation techniques exceeds the scope of this manual, so we shall limit ourselves here to a brief overview. The first point to be made is to collect under proper sanitary conditions and to keep the semen (concentrated semen fraction) at a warm temperature. Then it must be examined under a microscope to verify sperm viability, motility and freedom from deformities. All observations must be properly recorded. It is not routine practice to take a careful reading of sperm density (although it would be best if such a measurement could be done), but if the density appears extremely low, it is safer not to use it. The Modena diluent, which has been warmed to
the same temperature as that of the semen, is poured into the semen collection bottle holding the sperm. The Modena is poured so that it runs down the side of the bottle. At the same time, the sperm sample which is already in the bottle is stirred gently as the pouring proceeds. The amount to be poured depends on the number of times the resulting solution is to be used, but should usually be about one to two times the amount of semen (resulting in a dilution ratio of two or three). For storage, a cooler set to 15 °C is used. Although there are individual differences between sires, these sperm can usually be used for one week as long as they retain their viability and motility when warmed up. Their use is not known to result in a lowered production of fertilized embryos. If the sperm are placed in the cooler without being used for one day or longer, the decrease in viability can be avoided by stirring the sample once per day.

Table III-3. Composition of Modena

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>27.5 g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>6.9 g</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>EDTA-Na₂</td>
<td>2.35 g</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.9 g</td>
</tr>
<tr>
<td>Trisaminomethane</td>
<td>5.65 g</td>
</tr>
<tr>
<td>Polymixin B sulfate</td>
<td>0.0167 g</td>
</tr>
<tr>
<td>Gentamicin sulfate</td>
<td>0.150 g</td>
</tr>
</tbody>
</table>

The reagents listed above are weighed and mixed with distilled water to make up 1,000 ml of Modena solution. It is stored at 4 °C and warmed when it is to be used. There is no special need to carry out sterilization by filtration.

8. Transport and starvation of embryo donors

The embryo donor must be starved for one day prior to surgery, and if possible the sow should not be allowed water during the 2 h prior to surgery.

9. Handling of the embryo donor after surgery

After surgery, the embryo donor must be kept in a clean pen or stall. She should be fed half the normal amount of feed on the day following surgery, and regulated thereafter as dictated by the progress of the sow’s recovery. In addition, appropriate antibiotics should be administered in order to prevent post-surgery infections. If a sow that has been treated with drugs is to be sent to the slaughterhouse, a legally mandated withdrawal period applies. If the same embryo donor is to be used repeatedly for embryo collection, pay close attention to the sow’s body condition and regulate the
amount of feed accordingly. Be especially careful not to let the sow grow too fat. Once surgery is finished, resume monitoring for signs of estrus. After an embryo donor has been through surgery, it is not uncommon for estrus to fail to resume, or for it to resume only after a long time. In such cases, resumption of estrus can be induced by bringing the sow into contact with a boar, or by administering eCG. When not all embryos have been collected from the sow, those remaining in the uterus may occasionally result in pregnancy. We must be alert to this possibility.

Further suggested readings

- Varley MA. Synchronization of oestrus in gilts. Pig news and information 1983; 4, 151-156.
Chapter IV. Preparation of the Embryo Recipient

The sow to which embryos are transferred is called the recipient sow or the embryo recipient. In order to perform embryo transfer, the estrous cycles of the embryo donor and embryo recipient must be at roughly the same point. In other words, their estrous cycles must be synchronized. In this chapter, we shall describe the preparation of the embryo recipient before embryo transfer, as well as the handling of the recipient after transfer. When a prepubertal gilt is used, it should be as close as possible to puberty (six to seven months old). If a mature sow is used, it is important for a successful embryo transfer that its estrous cycle be clearly established, and that this cycle occur on a regular basis.

<Required equipment and reagents>

<table>
<thead>
<tr>
<th>Product</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCG: Equine chorionic gonadotropin [eCG; formerly known as pregnant mare's serum gonadotropin (PMSG)]</td>
<td>Induction of estrus in embryo donor</td>
</tr>
<tr>
<td>hCG: Human chorionic gonadotropin (hCG)</td>
<td>Induction of estrus in embryo donor</td>
</tr>
</tbody>
</table>

1. Procedures for preparation of recipient pigs

The procedures for preparation of the embryo recipient are outlined in this section. First, an embryo transfer plan is formulated in accordance with the use to which the embryos are to be put. Then a recipient is selected, estrus induction procedures are performed, the recipient is monitored for signs of estrus, and the transport and starving of embryo recipients are carried out.

![Diagram of procedures for preparation of recipient pigs](image)
2. Embryo transfer plan

In formulating an embryo transfer plan, it is necessary to take into account such factors as the time and method of embryo transfer, the number of embryo recipients, the necessary disease-control considerations, and the method of estrus induction.

3. Points to consider in selecting an embryo recipient

When selecting an embryo recipient, the following points should be considered. The number of embryos involved and the use to which the embryos will be put are especially important.

① The recipient should satisfy all disease-control requirements for the use to which the embryos are to be put.
② The recipient must be physically fit for the purpose of parturition and nursing, and its mammary glands must not be deficient in any way.
③ The recipient must satisfy all requirements for induction of estrus.
④ The recipient must be healthy enough to undergo surgery.
⑤ The recipient must have been vaccinated against certain diseases (Aujeszky's disease, Japanese encephalitis, parvovirus infection) which may cause abortion or mummified fetuses during pregnancy.

4. Estrus synchronization

To achieve a successful embryo transfer (i.e., to transfer embryos which later develop into piglets), it is necessary to synchronize the estrous cycles of the embryo donor and recipient. The pregnancy rate tends to be higher when the estrous cycle of the recipient lags one to two days behind that of the donor (see Table IV-1). Estrus induction should be carried out with the aim of establishing a one-day lag. While the degree of synchronization of estrus is usually gauged on the basis of the beginning of estrus (standing heat), the timing of ovulation is related to the end of estrus, and the duration of estrus varies according to estrus induction techniques and the individual pig. Ovulation times can differ even when estrous cycles begin at the same time, so the donor and recipient must be monitored until the end of estrus. When embryos are to be transferred in the initial stages of development (1- or 2-cell stage), it is common to opt for complete synchronization of the estrous cycles of the donor and recipient.
Table IV-1. Estrous cycle synchronization and corresponding pregnancy rates

<table>
<thead>
<tr>
<th>Degree of estrous cycle synchronization</th>
<th>No. of studies upon which figures are based</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2</td>
<td>1</td>
</tr>
<tr>
<td>+1</td>
<td></td>
</tr>
<tr>
<td>±0</td>
<td></td>
</tr>
<tr>
<td>-1</td>
<td></td>
</tr>
<tr>
<td>-2</td>
<td></td>
</tr>
</tbody>
</table>

Pregnancy rate
(1/22) 5% 47% 71% 78% 86%
(14/30) (22/31) (28/36) (18/21)

Pregnancy rate
--- 40% 55% 74% 72%
--- (2/5) (22/40) (46/62) (36/50)

*: +2 ; Recipient begins estrus 2 days before donor. +1 ; Recipient begins estrus 1 day before donor. ± 0 ; Recipient begins estrus on same day as donor. -1 ; Recipient begins estrus 1 day after donor. -2 ; Recipient begins estrus 2 days after donor.

5. Methods of inducing estrus

If one intends to use natural estrus for the embryo recipient, it is necessary to raise a very large number of sows and monitor them carefully for estrus. This method is, therefore, not very efficient. There are five main estrus induction methods, listed as items a. through e. in the inset below. The standard eCG dosage is 1,000 IU per head for each of these methods, but the reaction of the ovaries to eCG varies depending on such factors as the season, the type of eCG, its lot number, differences between individual sows, differences between breeds, etc. When a sow is relatively unresponsive to eCG, the dosage should be increased to 1,250 IU or 1,500 IU. On the other hand, if administration causes excessive ovulation or cystic corpora lutea, the dosage should be reduced to 750 or even 500 IU. Except for the fact that mating and artificial insemination are not required, the basic methods for induction and monitoring of estrus in the embryo recipient are exactly the same as those for the embryo donor. These have already been described above, so we shall not repeat the description here.

Methods of inducing estrus in pigs

a. Administration of eCG and hCG to a prepubertal gilt.
b. Administration of either PGF₂α or an analogue (PGF₂α-A) to a pregnant sow to induce abortion and make use of the subsequent estrus.
c. Utilization of the estrus which follows weaning.
d. Oral administration of a synthetic progestin.
e. Utilization of the natural estrous cycle.
6. Handling of the embryo recipient after surgery

After surgery, the embryo recipient must be kept separate from other pigs in a clean pen or stall. A pregnant sow is usually kept in a stall. This yields greater success in farrowing performance than when the pregnant sow is housed in a group pen. It is also easier to monitor pregnancy and the sow's body condition. When it is not possible to keep the sow in an individual stall, she should be kept with the same group of pigs throughout pregnancy. One-half the normal amount of feed should be fed on the day following surgery, and regulated thereafter as dictated by the progress of the sow’s recovery. In addition, appropriate antibiotics should be administered to prevent postsurgery infections. Once surgery is finished, resume monitoring for signs of estrus. In the 3rd week after surgery, or some appropriate point thereafter, check for pregnancy.

Cited references


V. Surgical Procedures

Embryos are collected from cattle by means of uterine flushing via the cervical canal, but this method is very difficult with pigs, in which a very long uterine horn (1.2 - 1.5 m) winds along a longitudinal course into the abdominal cavity. It is possible to perform non-invasive uterine flushing via the cervical canal on pigs with surgically shortened uteri, but sows which have undergone this procedure cannot be used for regular breeding. For this reason, there is no choice but to use surgical procedures for embryo collection. This is done by making an incision along the midline of the abdomen to expose the oviducts and uterus. Surgical collection of pig embryos must be done by means of a laparotomy operation.

We shall now describe the procedures for a laparotomy carried out by one surgeon and one assistant.

<Surgical facility>

- Operating room.
  1. Room temperature must be kept at no less than 25°C all year round, and the floor must drain properly.
  2. Walls must be insulated and treated to prevent condensation.
  3. Operations are more convenient if the area for inspection of collected embryos is next to the holding pen.

Figure V-1. Arrangement of operating room and surgical equipment

Photo V-1. Holding pen next to operating room (Tottori Swine and Poultry Experimental Station)

Photo V-2. Inside the operating room
V. Surgical Procedures

- Operating table: There are various types of operating tables. Shown in the photos below are a movable and adjustable table (Nagano Prefectural Animal Experimental Farm), a fixed adjustable table (Ministry of Agriculture, Forestry and Fisheries, Animal Experimental Farm), and a movable table that is not adjustable (Ministry of Agriculture, Forestry and Fisheries, Ibaraki Experimental Farm). If the table being used is not adjustable, some means of lifting the pig onto the operating table is required.

- Photo V-3. Movable and adjustable table
- Photo V-4. Fixed adjustable table

- Photo V-5. Movable table that is not adjustable
- Photo V-6. Movable and adjustable table (Y.S. New Technology)

- Surgery light: These lights come in many different configurations. They may be fixed to the ceiling or mounted on casters, for example, and the number of light bulbs/tubes varies. Select the type best suited to your own facility.
V. Surgical Procedures

<Required equipment and drugs>

<table>
<thead>
<tr>
<th>Product name</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine sulfate ¹</td>
<td>Used for preanesthetic medication (to inhibit secretions by the mucous membranes of airway, etc.; see Chapter XII)</td>
</tr>
<tr>
<td>Acarpane (Stressan®) ²</td>
<td>Used for preanesthetic medication (as a sedative; see Chapter XII)</td>
</tr>
<tr>
<td>Xyazine (Hypnovel®)</td>
<td>Used for preanesthetic medication (as a sedative, painkiller, muscle relaxant, etc.; see Chapter XII)</td>
</tr>
<tr>
<td>Ketamine (Ketalar®)</td>
<td>Used for preanesthetic medication (as a sedative, muscle relaxant, etc.; see Chapter XII)</td>
</tr>
<tr>
<td>Inhaler (1 set)</td>
<td>Used for general anesthesia</td>
</tr>
<tr>
<td>Isoflurane (Forane®) ⁴</td>
<td>Used as inhaled anesthetic</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Used for general anesthesia (can also be used in conjunction with nitrous oxide) Facilitates inhalation of anesthetic</td>
</tr>
<tr>
<td>Inhaler mask</td>
<td>The inhaler mask is made by cutting a large plastic bottle in half and using the upper section. The mouth of the bottle is attached to a rubber hose, and approximately six holes are pierced in the bottle near the edge where it was cut in half. (See Figure V-2 on the left.) A mask to anesthetize large dogs can also be used.</td>
</tr>
<tr>
<td>Tape</td>
<td>To hold the mask in place</td>
</tr>
<tr>
<td>Soda lime</td>
<td>To absorb carbonate gases (color shows when it is time for replacement)</td>
</tr>
<tr>
<td>Sutures ⁵</td>
<td>For suturing. The following types, which come in a cassette, are recommended for ease of use.</td>
</tr>
</tbody>
</table>

Catgut No. 0, for suturing the incision made in the uterus

Lyophilized (Kawasaki Seibutsukagaku Kenkyusho Co., Ltd.)

Nylon sutures for suturing the peritoneum and blood vessels

Suprion (Kawasaki Seibutsukagaku Kenkyusho Co., Ltd.)

Nylon sutures for suturing the linea alba and adipose layer

Suprion (Kawasaki Seibutsukagaku Kenkyusho Co., Ltd.)

Catgut for suturing of adipose tissue and skin

Lyophilized (Kawasaki Seibutsukagaku Kenkyusho Co., Ltd.)

Sterilization bin ⁴ For autoclave sterilization. Items below are listed in reverse order, with the first in the list placed on the bottom of the stack.

Fenestrated cloth or drape

Used to cover exposed uterus.

Tray with stainless steel lid

For storage of surgical instruments (should be covered with cloth after surgical instruments are put away)

Towel forceps

Scalpel blade holder

Suture holder

Suture needles ⁵ For suturing. The following four are wrapped in aluminum foil.

Short (high-curved) round needle For suturing the incision made in the uterus (not needed for an embryo transfer operation).

Short (high-curved) cutting needle For suturing the peritoneum and blood vessels.

Medium (low-curved) cutting needle For suturing the linea alba and fat layer.

Long (low-curved) cutting needle For suturing skin.

Kocher forceps (named after German Surgeon)

For holding an incision (4 pcs.)

Rochester-Pean hemostatic forceps

To perform hemostasis and hold the peritoneum (4 pcs.)

Scissors

For cutting the linea alba and peritoneum.

Scissors for ophthalmic use (sharp tips) For cutting a hole to insert a catheter.

Hooked tweezers For suturing of skin, etc.

¹ Use either acarpane or xylazine. ² Isoflurane is safer than halothane.

A halothane expander can be used (see Chapter XII). ³ Commercially available disposable needles that come with sutures can also be used. One example is Coated VICRYL (12-0 coated needle, 36 mm; absorbable synthetic suture, purploid made by Ethicon). ⁴ Another method of sterilization is to place the material into a two-ply paper feed bag, hold the top and tape it shut, and use autoclave sterilization. ⁵ See the section on sutures.
V. Surgical Procedures

- Surgical cloth or drape
  - For covering the pig (4 pcs; each drape placed two layers deep).
- Surgical clothing
  - Should include a surgical mask. (The gown, mask, etc. should be folded so that the side which is to be on the outside when worn is stored facing the inside.)
- Surgical cap
- Set of two surgical drapes
  - Covers the instrument table.
- Ropes for restraining front and hind limbs
  - Restrain the front and hind limbs (4 pcs, approx. 150 cm each).
- Plastic foot covers
- Soap
- Brush for use on pig
- Razor with replaceable blade
- Brush for surgeon's fingers
- Hibiscrub
- Hibitane®
- Wash bucket
- Iodine spray
- 70% alcohol spray
- Diluted tincture of iodine spray
- Cotton with 70% alcohol
- Sterilized disposable surgical gloves
- Tampon (wrapped absorbable cotton, about the size of a flat)
- Hizegaure®
- Epinephrine
- 1% chondroitin sodium sulfate
- Sterilized disposable blades
- Instrument table
- Paper towels
- Ampicillin
- Sterilized syringe
- Neutral detergent
- Laundry detergent
- Forms for recording observations
- Note pad
- Writing instrument
- Instruments for flushing of reproductive organs

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6) Better ones are used for operating on humans. Examples: Cera, brand, adhesive-coating membrane.

See Chapters VI and VII.
1. Pre-surgery preparations

(1) Starvation, preanesthetic medication, restraining the animal, etc.

① The two feedings prior to surgery are withheld (no water allowed during 2 h prior to surgery).
② The pig is transported to the operating room.
③ The pig is cleaned. Special care is taken in cleaning the legs.
④ Atropine sulfate (0.05 mg/kg) and either azaperone or xylazine (2 mg/kg) are administered via intramuscular injection, then 10-15 min later ketamine hydrochloride (10-20 mg/kg) is injected.\(^7\)
⑤ After the pig’s movements have been slowed through preanesthetic medication, the inhaler is used to induce anesthesia. The pig is then laid face up on the operating table.
⑥ The hind legs are tied to the operating table, while the forelegs are tied together in front of the pig’s chest.

(2) Anesthesia

① Draw the tongue from the angle of the mouth, then place the mask over the snout so that the pig breathes through the mask (see Photo V-7).
② Tape the tongue and mask to the snout to ensure that the tongue does not retract into the mouth and the mask does not slip out of place (see Photo V-8).

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\(^7\) Because ketamine does not act on norepinephrine, it must be used in conjunction with either azaperone or xylazine. Ketamine dosage can be reduced by intramuscular injection of butorphanol (0.5 mg/kg). In view of their pharmacological actions, it is often recommended in the United States that atropine, xylazine, ketamine, and butorphanol be administered together as a mixture. (See the notes at the end of the chapter for further information.)
3. Open the regulator on the anesthesia machine. To induce anesthesia, set the oxygen concentration to 1% and the isoflurane (or halothane) concentration to 4%.

4. For maintaining anesthesia, set the oxygen concentration to 1% and the isoflurane (or halothane) concentration to 1-2% (isoflurane should start at 2% and be raised gradually to 4%).

(3) Cleaning and disinfection of the pig

1. Clean the surgical field on the pig (from the chest to the hind legs), with the operating table in the lowered position.
2. Shave the surgical field as large as possible.
3. Soak the pig down and brush vigorously three times. Rinse with water after each brushing.
4. Apply Hibiscrub and brush vigorously three times. Rinse with water after each brushing.
5. Apply diluted halothane to the surgical field.
6. Plastic foot covers have been soaking in a Hibiscrub solution. Place one over each of the left and right forefeet, and similarly over the hind feet.
7. Spray iodine on the surgical field.
8. Spray alcohol on the surgical field.
9. Adjust the height of the operating table to the height of the surgeon.
10. Lower the pig's head and keep the surgical field horizontal.
11. Spray alcohol on the top surface of the instrument table and wipe it with a paper towel.

(4) Preparation of the surgeon and assistant

1. Surgeon (S) and assistant (A): Clip fingernails.
2. S: Roll the sleeves above the elbow. Apply soap to the fingers, hands, and forearms and brush thoroughly three times, including the fingernails. Rinse with water after each brushing. (Do not touch the water faucet during this procedure). Next, apply to the surgeon's hands and forearms the same Hibiscrub that has been used to scrub the surgical field, and brush the hands and forearms thoroughly three times (it is easiest to use a foot-operated or elbow-operated dispenser). Rinse with water only after the first two brushings. After the third brushing, the surgeon holds out both hands in front of the assistant.
3. A: Spray iodine and then alcohol onto the surgeon's hands and forearms.
4. S: Hold both hands up and wait.
5. A: Wash both hands and open the canister holding the surgical garments. (If it is a paper bag rather than a canister, open the paper bag without touching the inside of the bag, and hold it so that the surgeon can see into the bag and remove the contents.)
6. S: The first items removed will be two cloth drapes used to cover the instrument table. Spread these over the instrument table, then remove the rest of the items, including the surgical gown and cap, and place them temporarily on the instrument table.
7. S: Put on the cap, surgical gown, and mask. (When putting on the cap, take care not to touch your hair. When putting on the mask, make it easy for the assistant to be the strings together by grasping the left and right strings in the middle.)
8. A: Stand behind the surgeon and tie the strings on the cap, gown, and mask. (When tying the strings on the mask, be careful not to touch the surgeon's fingers.) [See Photo V-9 on the next page.]
9. S: Place the four surgical drapes on the pig in a ring around the surgical field (15 cm x 5 cm; see V-2), with one to the left of it, one to the right, one in front, and one behind. [See Photos V-10 and V-11 on the next page.]

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5. Surgical Procedures

10. S. Open the stainless steel tray and, using the towel forceps, grip the pig's skin through the surgical drapes at each of the four corners of the surgical field.

11. A: While the surgeon is performing steps 9 and 10:
   - Control the concentration of the inhalant in accordance with the pig's state of anesthesia.
   - Place the ampule of Chondron in the temperature-controlled water bath to warm it.
   - Pour another ampule of Chondron into the bottle of physiological saline.
   - Without touching the contents, open the packages containing the surgical gloves, blade, tampons, and hizegauze, and place the packages in the lid of the tray on the instrument table.

12. S: After putting on the surgical gloves, load the scalpel blade onto the blade handle.

13. A: Hand the sutures (for suturing blood vessels) to the surgeon without touching them.

14. S: Receive the sutures from the assistant.

[This completes preparations for the laparotomy operation.]

2. Operation

1. Division of labor between surgeon and assistant

   In principle, the surgeon is the only one who touches the pig. The assistant keeps a record of the progress of the surgery (time when surgery begins, etc.); assists with anesthetic management and flushing of the embryos; helps the surgeon to perform hemostasis, cut sutures, and administer drugs; applies physiological saline to the uterus; etc. After flushing, if there is a need to search for the embryos in the operating room, this is also done by the assistant. For information on how to handle the embryos, see Chapter VIII.

2. Laparotomy operation

   a. Cutting into the skin

   1. Beginning either immediately above the pubic bone or at the next-to-last pair of teats, make an incision in the skin along the midline (As a general rule, the incision should be at least 15 cm long,
beginning at the last pair of teats in pigs weighing 100 kg or less, and beginning at approximately 5 cm below the last pair of teats with a length of 15 cm or more in pigs weighing more than 100 kg. Until the surgeon is thoroughly familiar with laparotomy operations, it is best to make the incision as long as possible in order to avoid subjecting the uterus and ovaries to undue force when pulling them out).

![Photo V-12. Cutting the skin](image)

2 Use a scalpel to cut down to the subcutaneous tissue, avoiding blood vessels (or tying them as the incision is made).

(b) **Hemostasis**

1 When a small blood vessel has been incised, it should be grasped with Rochester-Penan forceps and either twisted (by the surgeon) or have Bosmine poured over it (by the assistant).
2 When a large blood vessel has been incised, it should be sutured with nylon in advance (if the nylon sutures come in a cassette, help from the assistant is required).

(c) **Cutting into the adipose tissue**

1 Using the left thumb and the three middle fingers of the right hand, spread apart the transverse adipose tissue immediately below the subcutaneous layer, where the incision has been made. If the transverse adipose tissue is difficult to spread, cut it with a scalpel. Repeat this process until

![Photo V-13. Spreading apart the adipose tissue](image)

![Figure V-3. Schematic diagram of location of incision](image)

![Figure V-4. Structure of the incised adipose tissue, viewed from above](image)
the adipose tissue has been spread apart all the way to the upper end of the incision. In cutting through the transverse adipose tissue, cut down to the upper part of the longitudinal adipose layer (this method makes it possible to find all relatively large blood vessels (mammary veins) through visual inspection, enabling the surgeon to avoid cutting one by mistake).

2 When you have reached the longitudinal adipose layer, which can be spread apart without the aid of a cutting instrument, use the left thumb and the three middle fingers of the right hand to spread it apart. The muscular structure along the midline (the circled part in the figure below) can now be examined by pressing lightly with a finger on the the middle of the incision (the midline) and moving it left and right. Aiming for this spot, spread apart the longitudinal adipose layer until you reach point A, then continue spreading tissue with your fingers in the same manner until you reach point B. Expose the linea alba without moving the midline out of place. When you reach point B, the midline will be visible as a whitish and transparent area.

Figure V-5. Cutaway view from last teat to next-to-last teat

Figure V-6. Cutaway view of midline

(d) Cutting into the linea alba 6 (aponeurosis)

1 Grasp with forceps the two places marked with a ●, and then use a scalpel to make a short incision in the center (marked with a straight black line). If the incision is made in the center (the proper location for an incision), adipose tissue will appear. If the incision is made off center, a straight abdominal muscle will appear (the two straight abdominal muscles are about 5 to 10 mm apart).

Figure V-7. Schematic diagram of open incision to linea alba

Figure V-8. Schematic diagram of open incision to peritoneum

Figure V-9. Open incision of linea alba
2. Once adipose tissue has been confirmed through the incision, extend the incision along the midline to a length of ca. 10 cm.
3. Use Kocher forceps to spread the abdominal wall apart as shown in Figure V-8.

(e) Cutting into the peritoneum (serous membrane)
1. The adipose layer along the midline is thin. When parted with the fingers, the uterine horns and ileum can be seen underneath the thin peritoneum.
2. Using a pair of Kocher forceps, grasp the surface of the peritoneum in corresponding positions on either side of the midline, perforate the center with a scalpel, and use surgical scissors to make an incision of a little less than 10 cm along the midline (almost no bleeding occurs at this stage).
3. Release the Kocher forceps holding the aponeurosis and grasp the aponeurosis again together with the peritoneum. The incision should end up as shown in Figure V-8 and Photos V-15 and V-16.
4. Next, if the purpose of the operation is collection and transfer of embryos, the uterus is pulled from the abdominal cavity. Before doing this, remove all but two of the Rochester-Pean forceps holding the peritoneum, and return them to the stainless steel tray.

Photo V-15. Cutting into the peritoneum

Photo V-16. Intestines can be seen through the incision in the peritoneum.

(3) Exposing the uterus
(a) Wash the fingers
1. A: Prepare a quantity of either physiological saline or Ringer's solution and pour it over the surgeon's fingers (regardless of which one is used, it should contain Chondron, and shall be referred to hereafter as physiological saline).
2. S: Use the physiological saline poured by the assistant to wash off blood from the fingers.

---

8) Linea alba (aponeurosis): The linea alba (Latin for "white line") is where the aponeuroses of the abdominal muscles (external oblique muscle, internal oblique muscle, transverse muscle of abdomen) intersect, and it also contains longitudinal fibers. It has very few blood vessels giving it a whitish color, hence its name. (See the explanation of terms on page 42)
V. Surgical Procedures

(b) Setting the fenestrated cloth or drape

1. S: Set the fenestrated drape over the pig so that the surgical field shows through the opening in the cloth.
2. S: Set the Hizegauze over the fenestrated drape.
3. A: Pour physiological saline so that the gauze is thoroughly moistened (see Photo V-17).

(c) Exposing the uterus

1. With the palm of the hand facing upward, feel for the uterus with the middle and index fingers (the intestines have a thin membrane and feel very soft, while the uterine horns are firmer, extremely elastic, and feel harder than the intestines). Grasp the mesometrium between two fingers and pull it out onto the gauze. Handling the uterus with extreme care, pull out the uterine horns one at a time. In order to prevent adhesions while doing this, touch the uterine horns as little as possible, and be especially careful not to subject the oviducts or ovaries to undue force. When a uterine horn comes into view, grasp the mesometrium between the thumb and index finger and pull it out gently. When this procedure is followed, the uterine horns and mesometrium will not be subject to undue force, and it is possible to pull a fairly long part of the uterus through the incision in a single motion.
2. S: After extracting the uterine horns, determine which is the left and which is the right horn (see Photo V-18).

Photo V-17. The gauze placed over the fenestrated cloth is moistened thoroughly.

Photo V-18. The uterus is exposed through the incision, and the surgeon distinguishes the left uterine horn from the right.
(d) Exposing the ovaries

1. S: With the palm of the hand facing upward, use the middle and index fingers to pull out the ovaries, leaving each covered by its infundibulum. Perform an ovulation count (number of corpora lutea) and check the condition of the ovaries. If an infundibulum is separated from its ovary, gently place it back over the ovary (see Photo V-19).

2. A: Record the ovulation count and the condition of the ovaries.

3. S: After verifying the ovulation count, immediately place the ovaries back inside the abdominal cavity (except when an oviduct flush is to be performed).

(e) Preventing the exposed uterus from drying out

1. A: After the uterus has been exposed, prevent the surface of the uterus, oviducts, and ovaries from drying out by pouring liberal amounts of physiological saline on them.

(4) Embryo collection

There are two flushing methods for collecting embryos, the oviduct flushing method (for collecting early stage embryos) and the uterine horn flushing method. In this section we shall describe the latter method. Flushing is performed on one uterine horn at a time (see Chapter VI).

(a) Inserting the catheter

1. A: Hand a balloon catheter, ophthalmological scissors, and uterine incision sutures to the surgeon. Afterwards, continuously pour physiological saline on the uterus as long as it remains outside the abdominal cavity.

2. S: Use the ophthalmological scissors to pierce a hole in the uterus where the mesometrium attaches to the uterine horn, in a place where there are no blood vessels. Spread the scissors slightly to make an incision of ca. 5 mm in length (see Photos V-20 and V-21).

3. S: Insert the balloon catheter through the incision and maneuver it toward the tip of the uterine horn. Check the position of the balloon (see Photos V-22 and V-23).
(b) Infusion and recovery of the flushing fluid

1. A: Following the instructions of the surgeon, attach a syringe which has been filled with air to the air valve on the catheter, and inflate the balloon. Next, insert a blunt-tipped needle attached to a syringe into the lumen (opening) of the catheter and infuse 50 ml of flushing fluid into each of the uterine horns (see Photo V-24).

2. S: Infuse the flushing fluid into the tip of the uterine horns without generating excessive friction in the uterus and, with the fluid held at the utero-tubal junction, shake the uterine horns. Then, force all the flushing fluid into the catheter without putting excessive pressure on the uterine body (see Photo V-25).

3. A: Place the drain end of the balloon catheter into a 100 ml glass test tube and hold the test tube in a position lower than the uterus (see Photo V-26).

4. S: If it is difficult to recover all the flushing fluid, coax the fluid out by lightly rubbing the uterus near the intake hole on the catheter, by rotating the balloon, etc. (partly deflating the balloon sometimes facilitates recovery of the flushing fluid).

5. A: Following the instructions of the surgeon, deflate the balloon after the embryo collection procedure is completed.

6. S: With its tip pointing upward, gently remove the catheter.
V. Surgical Procedures

Photo V-26. Recovering the flushing fluid.

Figure V-9. Lembert suture (drawing on the right depicts a cutaway view) Source: Veterinary Surgery, Kanehara Publishing. Reproduced with permission.

7 A: Inject a small amount of flushing fluid into the catheter to wash out the inside of the catheter, and pour this fluid into the test tube containing the flushing fluid recovered previously. Give the catheter to the surgeon (if the second uterine horn has not yet been irrigated) and place the test tube with the recovered fluid in a temperature-controlled water bath (37-38.5°C). The fluid will be kept there until it can be examined.

8 S: Close the incision in the uterus with at least two Lembert sutures using catgut (see Figure V-9).

9 S: Clean any blood from the uterus using physiological saline.

10 S: After completing the flushing procedure on one uterine horn, place it back inside the abdominal cavity and pull out the other uterine horn. (Follow the same procedures as used to flush the first uterine horn.)

11 S: After the embryo collection procedure is finished (and also after an embryo transfer procedure is finished), check to be sure that no suture scraps, blood, or blood clots are still adhered to the uterus, place the uterus back inside the abdominal cavity, moving sequentially from one end of the uterus to the other. While doing this, be careful not to subject the uterus to undue force.

12 S: Remove the Hizegauze and fenestrated drape to the instrument table. Count the number of instruments and other items used to be sure that nothing has been left inside the abdominal cavity.

13 A: After the flushing procedure is finished, pour Chondron onto the uterus.

(5) Closing (suturing) the abdomen (procedure when sutures are packaged in a cassette)

(a) Suturing the peritoneum (serous membrane) and linea alba (aponeurosis)

1 A: Holding the cassette with nylon peritoneum suture in one hand, take the end of the suture with the other hand and pull out an appropriate length (approximately four times the length of the incision; same ratio applies elsewhere).

2 S: Use the left hand to grasp the pulled out suture material in the middle. Then, using scissors, cut off the tip held by the assistant, and cut the suture where it feeds from the cassette. (Prepare two sutures, one for the peritoneum and one for the linea alba. Prepare all other sutures in this same manner.)

3 A: Pour 50 ml of Chondron into the abdominal cavity.

4 S: Thread the peritoneum suturing needle. Two Rochester-Pean forceps are still holding the incision. Pick them up and suture together the left and right sides of the peritoneum, taking care not to damage any organs inside the abdominal cavity. (The stitch should be less than one finger in width.)

5 S: Suture continuously together the left and right sides of the linea alba. (It is also acceptable to use an interrupted suture for the peritoneum and linea alba.)
(b) Suturing the adipose layer

① S: Cut off an appropriate length of catgut suture for adipose tissue.

② S: Using a continuous stitch, close the adipose layer in the following manner. Insert the catgut into the subcutaneous tissue on one side of the incision, draw it out, and back in again until the needle (use a cutting needle) almost reaches the aponeurosis. Pull the needle out at point A (shown in the figure below), draw it lightly across the raised part where the aponeurosis and peritoneum have been sutured, insert it back into the adipose layer on the opposite side near the aponeurosis, draw it back out, and pass it through the upper part of the adipose layer once more before drawing it out from the subcutaneous tissue. (This procedure, which is illustrated in Figure V-9 below, prevents the occurrence of dead space.)

(c) Suturing of the skin

① S: Cut off an appropriate length of nylon skin suture.

② S: Use hooked tweezers to draw together the skin on the left and right sides of the incision, then use a low-curved needle to close the incision with a continuous mattress suture, as shown in the schematic drawing to the right.
V. Surgical Procedures

(d) Preventing infection
   ① A: Administer ampicillin via intramuscular injection (see instructions for the proper dosage). To prevent the ampicillin from leaking out after the needle is withdrawn, hold a finger over the point of injection and massage the surrounding area for a short time.
   ② A: Remove the surgical drapes and spray the incision area with diluted tincture of iodine.

(6) Post-operation procedures
   ① Count the surgical implements.
   ② Untie the pig's feet.
   ③ Set all gas concentrations to zero.
   ④ Remove the anesthetic mask.
   ⑤ Place the pig on a cart and move it to the recovery room.
   ⑥ Post-surgery cleanup
      • Recover any liquid anesthetic remaining in the vaporizer.
      • If the soda lime has turned purple, change it before the next operation is performed.
      • Remove the rubber balloon and flexible hose from the inhaler and allow the inside to dry out.
      • Wash the surgical instruments with a neutral detergent and dry them.
   ⑦ On the day following the operation, spray tincture of iodine over the incision area.

(7) Tips for preventing the occurrence of adhesions on the uterus, etc.
   ① Do not allow the uterus or other internal organs to become dry. The assistant must continually use physiological saline containing Chondron to keep internal organs moistened. (As a general rule, about 500 ml is used in operations that last 1 h or less.)
   ② Do not subject the uterus to undue physical stress.
   ③ Prevent bleeding from the abdominal wall, etc. Hemostasis should be thorough when bleeding occurs.
   ④ Make sure the flushing procedure progresses smoothly.
   ⑤ Keep the operation time as short as possible. (It is necessary to prepare for the operation in advance, and to work in close cooperation with the assistant.)

Cited reference

Further suggested readings
• Sugie T. Embryo Transfer of Livestock, Yokendo Publishing Tokyo 1989.
VI. Surgical Collection of Embryos

When collecting pre-implantation stage embryos from mammals, the most common method is to flush the oviducts and uterus with phosphate buffered saline (PBS). In this chapter we shall describe the surgical procedures for the collection of pig embryos (e.g., the oviduct flushing method, the oviduct/upper uterine horn flushing method, and the uterus flushing method), that are carried out after the uterus and ovaries have been exteriorized via surgical means. We shall also describe how one searches for the embryos after flushing procedures have been completed. There will be some repetition in the descriptions of these methods, but this cannot be avoided in an instruction manual.

Collect the embryos at a stage of development that is appropriate for the use to which the embryos are to be put. Many different methods are used. It is best to adopt those procedures which are most suited to one's own particular situation.

<Required equipment and reagents>

<table>
<thead>
<tr>
<th>Product name</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flushing fluid</td>
<td>For embryo flushing</td>
</tr>
<tr>
<td>Dulbecco's PBS (powdered synthetic culture medium; Gibco, Cat.No. 21600-010, Nissui, product number 05913)</td>
<td>Base fluid for flushing medium</td>
</tr>
<tr>
<td>Syringes (20 ml oviduct flushing, 50 ml uterine flushing)</td>
<td>For flushing</td>
</tr>
<tr>
<td>Injection needles (20 G: oviduct flushing, 18 G: uterine flushing)</td>
<td>For flushing</td>
</tr>
<tr>
<td>Dish with latticework grid lines. (Use injection needles to draw a grid lines on the back of a Falcon 1012 or 90 mm x 20 mm dish)</td>
<td>For examination of embryos</td>
</tr>
<tr>
<td>10 Fr. Nelaton's catheter (double-holed tip) or Teflon tube measuring 3.3 mm in outer circumference. (The length of either one is 30 to 40 cm.)</td>
<td>For oviduct flushing</td>
</tr>
<tr>
<td>18 Fr. two-way balloon catheter</td>
<td>For uterine flushing</td>
</tr>
<tr>
<td>Warming plate (warm plate: Sakura, PS-52 and others)</td>
<td>To keep the embryos warm</td>
</tr>
<tr>
<td>Incubator or temperature-controlled water bath</td>
<td>To keep the embryos warm</td>
</tr>
<tr>
<td>Stereomicroscope</td>
<td>For examination of the embryos</td>
</tr>
<tr>
<td>Clean bench</td>
<td>For preparation of flushing fluid</td>
</tr>
<tr>
<td>Stirrer</td>
<td>For weighing reagents</td>
</tr>
<tr>
<td>Top-plate counter scale</td>
<td>For preparation of flushing fluid</td>
</tr>
<tr>
<td>Pipettes for embryo manipulation</td>
<td></td>
</tr>
<tr>
<td>Super-purified water or deionized water that has been distilled twice</td>
<td>For preparation of flushing fluid</td>
</tr>
<tr>
<td>Sterilization filter (mesh: 0.22 micrometers)</td>
<td>To sterilize flushing fluid through pressurized filtration</td>
</tr>
<tr>
<td>Forms for recording observations</td>
<td>For recording observations</td>
</tr>
</tbody>
</table>
1. From the laparotomy operation to embryo collection

![Diagram showing the procedure: Perform laparotomy to expose uterus and ovaries, flush reproductive organs (oviducts, uterus), search for embryos.]

Figure VI-1. Procedures from the laparotomy operation to embryo collection

2. Interval after mating of embryo donor, and the organ selected for flushing

The embryo donor begins to ovulate about 40 h after hCG administration. The ovulated oocytes migrate to the ampulla of the uterine tube, where they come into contact with sperm and are fertilized. About 40 h after ovulation, 1- to 4-cell embryos can be collected by irrigating the oviducts. Otherwise, the embryos will collect in the upper part of the uterine horns (approximately the lower 30 cm, beginning at the tips) by the 5th day after mating. By flushing here, embryos can be collected at anywhere from the 4-cell to the blastocyst stages. By the sixth day after standing heat, however, the blastocysts sometimes migrate to the lower uterus so, beginning from the sixth day after standing heat, the entire uterus must be flushed. Table VI-1 (next page) lists the developmental stages of the embryos and the appropriate place to flush at different intervals following ovulation.

3. Preparation of the flushing fluid

The fluid used for flushing of the oviducts or uterus should include various additives, such as a culture medium (phosphate or Hepes buffer), inactivated fetal calf serum, albumin and/or other proteins, and such antibiotics as penicillin and streptomycin. In this chapter we will describe the preparation of a flushing fluid with a base medium made from Dulbecco's PBS\(^1\) and M-2\(^2\), both of which are very widely used in Japan. The water used to prepare the flushing fluid should be either a super-purified water (such as Milli-Q Plus, Millipore® Lab Products) or deionized water which should be distilled twice. Dulbecco's PBS is sold commercially in powdered synthetic form (Gibco, Cat.No. 21600-010), and this product works very well as a base medium.

After the flushing fluid has been prepared and sterilized, pour it into culture bottles with caps. The size of the bottles depends on the amount to be used. Place it in an incubator or a similar device and warm it to 37°C at 2h to 3h before it is scheduled to be used. After the flushing fluid has been sterilized, it can be preserved for a while if it is placed in a refrigerator.
Table VI-1. Developmental stages of pig embryos and the appropriate place to flush at different intervals following ovulation

<table>
<thead>
<tr>
<th>Interval (h) after injection of hCG</th>
<th>Stage of the embryo</th>
<th>Site flushed</th>
</tr>
</thead>
<tbody>
<tr>
<td>48-56</td>
<td>Pronuclear stage</td>
<td>Oviducts</td>
</tr>
<tr>
<td>-65</td>
<td>1-cell embryos</td>
<td>Oviducts</td>
</tr>
<tr>
<td>40-84</td>
<td>1- to 2-cell embryos</td>
<td>Oviducts</td>
</tr>
<tr>
<td>-95</td>
<td>2-cell embryos</td>
<td>Oviducts and upper uterine horns</td>
</tr>
<tr>
<td>-110</td>
<td>4-cell embryos</td>
<td>Upper uterine horns</td>
</tr>
<tr>
<td>-116</td>
<td>8-cell embryos</td>
<td>Upper uterine horns</td>
</tr>
<tr>
<td>-158</td>
<td>Compact morulae</td>
<td>Upper uterine horns</td>
</tr>
<tr>
<td>-184</td>
<td>Blastocysts</td>
<td>Entire uterus</td>
</tr>
<tr>
<td>-188</td>
<td>Expanded blastocysts</td>
<td>Entire uterus</td>
</tr>
</tbody>
</table>

(1) Preparation of flushing fluid based primarily on Dulbecco’s PBS

The components of a flushing fluid based primarily on Dulbecco’s PBS are listed in Table VI-2 (next page). First, weigh all components other than calcium chloride and magnesium chloride, pour them into a container of super-purified water, and stir the mixture thoroughly with the stirrer. (The amount of water in the container should be 70% of the amount of flushing fluid required.) Next, pour the calcium chloride and magnesium chloride into a separate container of super-purified water (20% of the amount of flushing fluid required), and stir it thoroughly. (If the chemicals are not completely dissolved, the solution will appear cloudy.) Pour the solution of calcium chloride and magnesium chloride slowly into the previously prepared solution to mix them. After the two solutions are mixed together, transfer the resulting solution to a measuring flask and add super-purified water up to the required amount of flushing fluid. Add blood serum, or blood serum albumin or other proteins, to the flushing fluid, and sterilize it with a 0.22 μm sterilization filter.

(2) Preparation of flushing fluid based primarily on M-2

The components of an flushing fluid based primarily on M-2 are listed in Table VI-2 (next page). M-2 is a culture medium, and is the same as Krebs-Ringer solution except that part of the bicarbonate has been replaced with Hepes buffer. First, weigh all components other than calcium chloride and magnesium sulfate, pour them into a container of super-purified water, and stir it thoroughly with the stirrer. (The amount of water in the container should be 70% of the amount of flushing fluid required.) Next, pour the calcium chloride and magnesium sulfate into a separate container of super-purified water (20% of the amount of flushing fluid required), and stir it thoroughly. (If the chemicals are not completely dissolved, the solution will appear cloudy.) Pour the solution of calcium chloride and magnesium sulfate slowly into the previously prepared solution to mix them. After the two solutions are mixed together, transfer the resulting
solution to a measuring flask and add super-purified water up to the required amount of flushing fluid. Next, add 1 N of HCl or NaOH to adjust the solution to pH 7.3. Then add serum, or albumin or other proteins, to the flushing fluid, and sterilize it with a sterilization filter. Passing the solution through a sterilization filter will raise the pH level by about 0.1. In addition, be aware that there are two types of HEPES. Hepes acid (FW = 238.3) and sodium salt (FW = 260.3). This must be taken into account when the components are weighed.

<table>
<thead>
<tr>
<th>Component</th>
<th>Dulbecco's PBS</th>
<th>M-2</th>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>800.0</td>
<td>553.5</td>
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<tr>
<td>KCl</td>
<td>20.0</td>
<td>35.6</td>
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<tr>
<td>CaCl₂/2H₂O</td>
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<td>MgCl₂/6H₂O</td>
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</tr>
<tr>
<td>MgSO₄/7H₂O</td>
<td>-</td>
<td>29.3</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
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<td>KH₂PO₄</td>
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<tr>
<td>NaHCO₃</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>HEPES* (Sodium salt)</td>
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<td>546.6</td>
</tr>
<tr>
<td>[(Hepes acid)]</td>
<td></td>
<td>500.4</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
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<tr>
<td>Sodium lactate (60% syrup)</td>
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<tr>
<td>1% phenol red</td>
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</tr>
<tr>
<td>Penicillin G (potassium salt)</td>
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<td>10,000 U</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
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<td>5.0 mg</td>
</tr>
<tr>
<td>Inactivated fetal calf serum</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>BSA (fraction V)</td>
<td>-</td>
<td>100 mg</td>
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</tbody>
</table>

* Either Hepes acid or sodium salt is used.

4. Flushing of the oviducts

In order to collect 1- to 4-cell embryos, which are found in the oviducts, oviduct flushing is carried out. There are two methods for flushing the oviduct. In the ascending method, the flushing fluid is injected into the oviduct from the uterus, and the embryos are collected from the oviduct where it connects to the infundibulum. In the descending method, the direction is reversed. In this section we shall describe the ascending method.
VI. Surgical Collection of Embryos

1. Count the number of ovulations and examine the condition of the ovaries and uterus.
2. Insert a catheter into the oviduct via the infundibulum.
3. Ligate the end of the catheter and place the end of the catheter into a dish with a grid.
4. Fill the syringe with flushing fluid.
5. Connect a 20 G needle to the syringe described in step 4.
6. Insert the needle described in step 5 into the tip of the uterine horn, and from there into the isthmus of the oviduct via the utero-tubal junction.
7. Inject the flushing fluid into the oviduct. It will collect in the dish.
8. Untie the catheter and pour the flushing fluid inside it into the dish.

The surgeon first checks the number of ovulations and examines the condition of the ovaries and uterus, and records all observations in the record book. Next, the tip of either a 10 Fr. Nelaton's catheter (the end with the holes) or a Teflon tube measuring 3.3 mm in outer circumference is inserted 4 or 5 cm from the infundibulum into the oviduct. Be careful not to insert it too far, since this sometimes lowers the collection rate. If a Nelaton's catheter is being used, ligate the catheter at ca. 2 cm from the tip. If a Teflon tip is being used, ligate the tube at ca. 1 cm from the tip. If the ligation is made too close to the oviduct, the oviduct could be damaged, causing leakage of flushing fluid. When the needle is passed through the mesosalpinx, bleeding will not occur if blood vessels are avoided. Also, instead of ligation, the assistant can use the fingers to pinch the cathether or tube at the ligation site. The end of the catheter or tube not inserted into the oviduct is placed by the assistant into the dish with the grid. Next, the surgeon fills the syringe with 20 to 35 ml of flushing fluid and connects a 20 G needle to the syringe. Taking care to avoid blood vessels, insert the needle into the tip of the uterine horn, and from there into the oviduct via the utero-tubal junction. With a finger

Photo VI-1. Oviduct flushing (knot is visible at center right)

Photo VI-2. Oviduct flushing (embryos collected directly into the dish)
placed over the tip of the needle, inject the flushing fluid slowly into the oviduct. It is possible to avoid accidentally poking the needle through the oviduct by cutting off the bevelled tip of the needle, thereby making it blunt. After the flushing of one oviduct has been completed, flush the other oviduct in the same manner. To keep the internal reproductive organs from becoming dry, and to prevent their adhesion to other tissues and to each other, apply physiological saline as needed during flushing. After both oviducts have been flushed, wash off all exposed reproductive organs with physiological saline. It is recommended that the saline contains chondroitin sodium sulfate (Product name: Chondron).

5. Flushing of the oviducts and upper uterine horns

By the second or third day after mating, 2- to 4-cell stage embryos will be located either in the oviducts or in the upper part of the uterine horns, or possibly in both places; hence, embryo collection at this point requires flushing of both of these locations.

Using either ophthalmological scissors or the tip of a pair of forceps, make a small incision about 40 cm from the tip of the uterine horn. To prevent bleeding, avoid blood vessels. Insert the tip of the balloon catheter through this incision 4 to 5 cm in the direction of the upper uterine horn. Next, insert a 10 ml syringe into the catheter’s inflation opening and inflate the balloon until it remains in a fixed position without moving. If the balloon is overinflated, it could cause bleeding in that area of the uterine

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1. Check the number of ovulations and examine the condition of the ovaries and uterus.
2. Insert the tip of a balloon catheter into the uterus at a point approximately 40 cm from the tip of the uterine horn, and be sure the catheter is pointed toward the tip of the uterine horn.
3. Inflate the balloon.
4. Fill the syringe with flushing fluid.
5. Place the end of the balloon catheter into a 50 to 100 ml test tube.
6. Connect a 20 G needle to the syringe described in step 4.
7. Insert the needle described in step 6 into the ostium abdominale.
8. Inject the flushing fluid.
9. Hold the tip of the uterine horn in a raised position and massage it while the flushing fluid drains into the catheter.
10. After the flushing fluid has been recovered, deflate the balloon.
11. If there is any flushing fluid remaining in the catheter, return it to the test tube.
12. Suture the incision (Lembert suture) that was made for insertion of the catheter.
VI. Surgical Collection of Embryos

horn, or could make it difficult to recover the flushing fluid. Also, when a balloon catheter is reused, air sometimes escapes through the inflation opening, so it is recommended that hemostatic forceps be used to pinch off the tube near the opening. The end of the catheter not inserted into the uterus is placed by the assistant into a 50 to 100 ml test tube. The surgeon then fills the syringe with 30 to 45 ml of flushing fluid, fits the syringe with a 20 G needle, and inserts the needle into the oviduct via the infundibulum. With a finger placed over the tip of the needle, inject the flushing fluid slowly into the oviduct. It is possible to avoid accidentally poking the needle through the oviduct by cutting off the tip of the needle, thereby making it blunt. After the flushing fluid has been injected, remove the needle from the oviduct, hold the tip of the uterine horn in a raised position, and massage the uterus gently (beginning at the tip of the uterine horn) while the flushing fluid drains into the catheter. Make sure the flushing fluid does not flow backward. It is often difficult to recover the flushing fluid. In such cases, tug lightly on the catheter and apply pressure to the fluid remaining in the uterus. After recovering roughly the same amount of flushing fluid that has been injected, deflate the balloon. If there is any flushing fluid remaining in the catheter, return it to the test tube and suture the incision that was made for insertion of the catheter. (Use a Lembert suture, described on page 49.) After the flushing on one side has been completed, flush the other oviduct and uterine horn in the same manner. To keep the reproductive organs from becoming dry, and to wash off any blood, apply physiological saline as needed during flushing. After flushing is completed, wash all exposed reproductive organs with physiological saline containing Chondron in order to prevent adhesions.

6. Flushing of the uterine horns

By the fourth or fifth day after mating, the embryos migrate to the upper uterine horns and, by the 6th day after mating, they are distributed throughout each of the uterine horns. Depending on when embryo collection is performed, either the upper portion or the entire area of each of the uterine horns must be flushed. The position at which the balloon catheter is inserted depends on the area to be flushed. Using either ophthalmological scissors or the tip of a pair of forceps, make a small puncture incision. To prevent bleeding, avoid blood vessels. Insert the tip of the balloon catheter through this incision 6 to 7 cm in the direction of the upper uterine horn. Next, connect a 20 ml syringe onto the catheter’s inflation valve or opening and inflate the balloon until it remains in a fixed position without moving. If the balloon is overinflated, it could cause bleeding in that area of the horn, or could make it difficult to recover the flushing fluid. Also, when a balloon catheter is reused, air sometimes escapes through the inflation opening, so it is recommended that hemostatic forceps be used to pinch off the tube near the valve. The free end of the catheter is then placed by the assistant into a 50 to 100 ml test tube. The surgeon fills the syringe with 50 ml of flushing fluid, fits the syringe
with an 18 G needle, and inserts the needle through the wall of the uterine horn near the
oviduct in the direction of the body of the uterus. With a finger placed over the tip of the
needle, inject the flushing fluid slowly into the uterine horn. It is possible to avoid
accidentally poking the needle through the uterus by cutting off the tip of the needle,
thereby making it blunt. Alternatively, the fluid can be injected into the uterine horn
directly through the catheter, and then withdrawn or drained back out via the same
route. It takes longer to flush by this method than it does to inject the flushing fluid
directly into the tip of the uterine horn, but it eliminates the need to insert a needle into
the tip of the horn. After the flushing fluid has been injected, remove the needle from the
uterus, hold the tip of the uterine horn in a raised position, and massage the uterus
gently (beginning at the tip of the uterine horn) while the flushing fluid drains into the
catheter. After recovering roughly the same amount of flushing fluid that has been
injected, deflate the balloon. If there is any fluid remaining in the catheter, return it to the
test tube and suture the incision that was made in the uterus. After the flushing on one
side has been completed, flush the other uterine horn in the same manner. To wash off
any blood from the reproductive organs, apply physiological saline as needed during
flushing. After flushing of both uterine horns is completed, before returning the
reproductive organs to the abdominal cavity, wash all exposed reproductive organs with
physiological saline containing Chondron in order to prevent adhesions.

1. Check the number of ovulations and examine the condition of the ovaries and uterus.
2. If only the upper uterine horns are to be flushed, insert the tip of a balloon catheter
   into the uterus at a point approximately 40 cm from the tip of the uterine horn. If the
   entire area of the uterine horns is to be flushed, insert the tip of a balloon catheter
   into the lower uterine horn (the end nearest the vagina).
3. Inflate the balloon.
4. Fill the syringe with flushing fluid.
5. Place the tip of the balloon catheter into a 50 to 100 ml test tube.
6. Connect an 18 G injection needle to the syringe described in step 4.
7. Insert the needle described in step 6 into the ostium abdominale.
8. Inject the flushing fluid.
9. Hold the tip of the uterine horn in a raised position and massage it while the flushing
   fluid drains into the catheter.
10. After the flushing fluid has been recovered, deflate the balloon.
11. If there is any flushing fluid remaining in the catheter, return it to the test tube.
12. Suture the incision that was made for insertion of the catheter.
7. Recovering embryos from the flushing fluid

When embryos are collected by flushing of the oviducts, the small amount of flushing fluid used can be collected into a dish which goes directly under a stereoscope for recovery of the embryos. It is best to search for and recover the embryos immediately after flushing has been completed, but if this cannot be done, it is also possible to collect the flushing fluid in test tubes and keep it in an incubator at 37°C. Use a Komagome type pipette or other such instrument to transfer the flushing fluid to a dish with a latticework grid. One 90 mmφ x 20 mm dish can hold all 50 ml of the flushing fluid. Use a pipette to retrieve the embryos from the dish and wash them three times by changing the culture medium into which they are placed. The embryos are washed most effectively if a different pipette is used each time.

Cited references

VII. Collecting embryos from a slaughtered sow

A pig does not have as high a monetary value as a cow; thus, slaughtering the sow before irrigating its oviducts and uterus is a frequently used alternative method of embryo collection. It is often reported, however, that due to autolysis of tissues, the flushings are extremely cloudy if not collected within 1 h after slaughter. An embryo collection procedure is generally considered quite successful if the recovery rate is 90%. When embryos are collected from slaughtered sows, there is a strong tendency for blood and debris to get mixed in with the flushing fluid, so an effort should be made to transfer the embryos to culture medium for washing as soon as possible after recovery. Studies have been carried out to compare the survival of embryos collected from slaughtered sows with those of embryos collected surgically from live sows\(^1\),\(^2\), but there is no clear proof of any difference. If the aforementioned precautions are observed, it is safe to assume that the survival is virtually the same.

<Required equipment and reagents>

<table>
<thead>
<tr>
<th>Product name</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene box</td>
<td>For transporting oviducts and uterus to the laboratory.</td>
</tr>
<tr>
<td>Plastic bag</td>
<td>Container for the oviducts and uterus. Label it in advance with sow's number, etc.</td>
</tr>
<tr>
<td>Scissors</td>
<td>For making incisions in the oviducts and uterus.</td>
</tr>
<tr>
<td>Warmer</td>
<td>Warmed and placed in transport box to keep the oviducts and uterus warm while they are being transported.</td>
</tr>
<tr>
<td>Surgical gloves</td>
<td>For trimming the oviducts and uterus.</td>
</tr>
<tr>
<td>Kimwipe</td>
<td>For washing the oviducts and uterus.</td>
</tr>
<tr>
<td>Tray</td>
<td>Receptacle for the oviducts and uterus.</td>
</tr>
<tr>
<td>Steel stand (three-legged, large size)</td>
<td>The uterus is hung from this stand during flushing. Equipped with clamps and muffs. Also holds the Em Con filter (see description in text).</td>
</tr>
<tr>
<td>Forceps (3 pairs)</td>
<td>For hanging the uterus up during flushing.</td>
</tr>
<tr>
<td>50 ml syringe</td>
<td>For injecting the flushing fluid.</td>
</tr>
<tr>
<td>PP catheter</td>
<td>For flushing of the oviducts. Total length is 14.5 cm; cut down to ca. 5 cm before use.</td>
</tr>
<tr>
<td>Flushing fluid</td>
<td>Should contain PBS and either serum or bovine serum albumin.</td>
</tr>
<tr>
<td>Em Con filter</td>
<td>Used in the final step of flushing fluid to recover embryos from the fluid.</td>
</tr>
<tr>
<td>Basin</td>
<td>Placed beneath the Em Con filter.</td>
</tr>
<tr>
<td>90 mm x 20 mm dish</td>
<td>Used to search for embryos. Before using, draw grid lines on the underside of the bottom.</td>
</tr>
<tr>
<td>Stereoscope</td>
<td>Used to search for embryos.</td>
</tr>
<tr>
<td>Clean bench</td>
<td>For preparation of culture medium, etc.</td>
</tr>
<tr>
<td>CO(_2) incubator</td>
<td>For culturing of embryos.</td>
</tr>
<tr>
<td>Culture medium</td>
<td>For culturing of embryos.</td>
</tr>
<tr>
<td>Sterilization filter</td>
<td>For sterilization of culture medium through pressurized filtration.</td>
</tr>
<tr>
<td>10 ml syringe</td>
<td>For filter sterilization of solutions.</td>
</tr>
<tr>
<td>Embryo manipulation pipette</td>
<td>For washing embryos. Made from a Pasteur pipette.</td>
</tr>
</tbody>
</table>
VII. Collecting embryos from a slaughtered sow

☐ Multiwell plate For culturing of embryos.
☐ Mineral oil To prevent evaporation of culture medium.
☐ 70% ethanol Kept in wash bottle, used to wash lab table, instruments, etc.
☐ Record book For keeping records.

1. Collection of oviducts and uterus

Immediately after the sow is slaughtered, quickly remove the ovaries, oviducts, uterus, and cervical canal (the position of the cuts is not critical) and place them in the plastic bag. Place the bag in the polystyrene box. If the weather is cold, place a heated warmer in the box.

2. Trimming the oviducts and uterus, and recording the number of corpora lutea

After the reproductive organs have been brought to the laboratory, remove them from the plastic bag and place them on the Kimwipe towel that has been laid over the tray. Thoroughly wash off any blood and cut off any parts not needed for the flushing procedure. Inspect the ovaries and record the number of corpora lutea, etc.

3. Flushing of the oviducts

Cut off the oviducts only, insert a section of PP catheter (cut to ca. 5 cm in length) via the infundibulum into an oviduct, and pinch it with the fingers to prevent fluid leakage. Inject about 20 ml of flushing fluid via the PP catheter, and recover it directly into a 90 mm dish.

4. Flushing of the uterus

It is reported that embryos migrate to the upper uterine horns by approximately the sixth day but, to be on the safe side, flush the entire length of each of the uterine horns one at a time.

1. Using forceps, grasp the uterine body and the mesometrium near the oviducts and hang the organ from the steel stand.
2. Make an incision of appropriate length in the myometrium and trim the part near the exit point in a straight line. After thoroughly wiping away any blood, cut the uterus near the uterine body. While pinching the cut to prevent any fluid inside the uterus from escaping, place the cut into the Em Con filter which has been installed beneath the stand.
3. There could be embryos located near the utero-tubal junction, so inject the first 20 ml of fluid via the infundibulum. Just as when flushing the oviducts, use a PP catheter cut to the appropriate length. The shape of the uterus is very serpentine, so massage the uterine horn from the tip toward the uterus while draining the flushing fluid downward. Recover the fluid via the Em Con filter. Next, use scissors to make an incision in the upper uterine horn, and use a syringe to inject 20 ml of flushing fluid directly into the uterine horn. Recover the fluid in the same manner as before (see Photo VII-1). An alternative method of embryo collection is to use a balloon catheter, just as in a surgical embryo collection procedure (see Photo VII-2).
VII. Collecting embryos from a slaughtered sow

4. Open the bottom clip to release the fluid into the basin below. Take care not to let the fluid overflow the Em Con filter. Keep the liquid flowing so that there is always some passing through the filter. After flushing is completed, if a small amount of recovered fluid is left on the filter and is washed through the filter with more flushing fluid, the recovered fluid will not turn cloudy, making it easier to find the embryos. Leaving a little fluid on the surface of the filter, transfer all the recovered flushing fluid to a 90 mm dish. Quickly pour a small amount of fluid through the filter to prevent the surface of the filter from drying out. Gently stir the filtered fluid and transfer it to the dish.

5. Finding the embryos

Before beginning the search procedure, draw latticework grid lines approximately 1 cm apart on the underside of the bottom of the dish. When collecting embryos from a slaughtered sow, there is a strong tendency for blood and debris to get mixed in with the flushing fluid, so the embryos should be transferred to culture medium for washing as quickly as possible.

Cited references

Further suggested readings
VIII. Handling of Embryos

1. Tips on embryo handling

Mammalian embryos develop in the aseptic, temperature-regulated environment of the oviducts or uterus. If good litters are to be obtained after transferring such embryos to the oviducts or uterus of another sow, it is necessary to control the factors that affect the survival of embryos while they are outside the sow’s body, and to avoid serious damage to the embryos’ developmental capacity. In other words, the environment in which embryos are handled must be as close as possible to that inside the sow’s body. However, we do not completely understand how embryos are controlled by the environment in vivo; thus, they must be handled under conditions which are highly reproducible. The following is a list of factors that affect survival of embryos outside the sow’s body:

| 1. Temperature       | 6. Presence/absence of proteins |
| 2. Light             | 7. Types/concentration of ions  |
| 3. Vibration         | 8. Type/concentration of energy source |
| 5. Hydrogen ion concentration | 10. Types/concentration of antibiotics |

The following precautions must be kept in mind when handling embryos outside the sow’s body.

(1) General precautions

If unidentified substances not required for embryo transfer are present when the embryos are handled, it is difficult to ensure a consistently uniform environment. Such substances should be kept out of the environment if at all possible. Contamination could occur in many ways. For example, instruments that come into direct contact with the holding medium might not be properly washed or sterilized; sterilized instruments might be handled improperly; the water used to prepare the culture medium might not be pure; a spoon for dispensing drugs might be contaminated; a reagent might be contaminated; blood serum or some other such substance might become denatured while in storage, etc. Also, there are many opportunities for contamination by bacteria during embryo collection and transfer, so it is necessary to be careful about maintaining aseptic conditions.

(2) Maintain aseptic conditions during embryo handling

The container which holds the embryos is not covered by a lid while recovered embryos are being searched for, washed, evaluated, stored, or manipulated. This increases the chances of contamination by bacteria, etc. Accordingly, it is necessary to
keep the laboratory in which embryos are handled as free as possible of bacteria and dust. It is also very important to maintain cleanliness of the fingers, hair, and clothing of those who handle the embryos. It is nearly impossible, however, to maintain a completely aseptic environment throughout the entire process, so it is necessary to add appropriate antibiotics to the culture medium. Also, persons who handle the embryos must always be careful about their own personal health.

(3) Avoid sudden changes in the environment

Sudden changes in the embryos' environment have a major impact on survival rates. Pig embryos are particularly sensitive to cold; thus, changes in temperature are thought to have the greatest impact. Irrigation fluid must always be kept at about 38 °C during flushing and after it is recovered. As well, it is highly recommended that the temperature of the room where embryos are collected and the laboratory where they are manipulated be kept at 25 to 30 °C all year round. Caution is required when embryos are kept in a Pasteur pipette with only a small amount of culture medium, because the temperature of the contents of the pipette can be affected very quickly by the ambient temperature. Although survival is not affected if embryos are subjected for a short time to temperatures higher than a pig's body temperature (42 °C for 3 h), it is best to avoid doing so unless there is a special reason for it. In addition, if a dish containing embryos in culture medium is placed on a warming plate without being covered by a layer of paraffin oil, the culture medium may partially evaporate. This affects the pH of the remaining medium, which in turn reduces the survival rate. Further, one should develop the habit of frequently checking the temperature of the temperature-controlled water bath and the warming plate. If the embryos are to be kept exposed to the ambient temperature for a while in a buffer solution based on carbon dioxide, HEPES or some other pH buffer should be added to the solution beforehand.

Embryo transfer techniques involve many procedures (including the manipulation of embryos outside the sow's body) that require a high degree of handling skill; thus, the proficiency of the surgeon and assistant has a great impact on embryo survival. For this reason, the surgeon and assistant must have a firm grasp of the theory, and must practice regularly in order to improve and maintain their skills. By doing this, they will be able to collect as many embryos as possible, handle the collected embryos skillfully without losing any, and carry out manipulation and transfer procedures without causing infertility.

2. How to search for embryos

In pigs, each uterine horn holds 50 ml of flushing fluid. Use a Komagome pipette to transfer all of the flushing fluid from a 100 ml test tube to a 90 x 20 mm plastic dish. Before transferring fluid to the dish, use a scalpel or other such instrument to cut latticework squares (ca. 1 x 1 cm) into the underside of the bottom of the dish to make it
easier to locate the embryos. Compared with cows, less mucus and debris gets mixed into flushing fluid recovered from pigs, which means that oocytes and embryos are easier to find. Continue searching for embryos until the number found at least matches the corpus luteum count. As embryos are found, transfer them as quickly as possible to a culture medium which contains blood serum, etc. Next, examine the embryos with an inverted microscope to determine the stage of development and their quality, and duly record all observations. It is best to photograph or sketch the embryos at this point, but finish this process as quickly as possible in order to avoid exposing the embryos to the light of the microscope for any longer than is absolutely necessary. Wash the embryos several times (as many as ten times). Pour 1 to 2 ml of culture medium into each well of a 24-well dish and place the embryos in the wells. Switch and wash pipettes after each embryo is transferred. A 0.25% trypsin solution (with the trypsin dissolved in Hank’s balanced salt solution) works very well as the culture medium. One of the main objectives of pig embryo transfer is to introduce new genetic materials into a herd without exposing it to disease. However, when transferring embryos to a recipient, it is especially important to wash the embryos properly and thoroughly. To wash the embryos effectively, it works very well if a gas burner is used to modify the suction tip of Pasteur pipettes so that their diameters match the size of the embryos.

3. Washing and sterilizing instruments

The purpose of washing with a water-based detergent solution is to increase the solubility of contaminants and dilute them to the point where all unknown soluble substances are removed from the object being cleaned. There are many ways to carry out washing; it is best to vary the method of washing depending on the instrument being washed, e.g.—what it is made of, its shape, what has contaminated it, and how dirty it is. For the purposes of washing, it is useful to classify the instruments used for embryo transfer into two types: those that come in contact, via culture medium, with the embryos, and those that do not. The method of washing instruments that come in contact via culture medium with the embryos is as follows.

(1) Pre-wash

Use tap water to wash unused glass instruments once or twice, then soak them overnight in a 1% hydrochloric acid alcohol solution to neutralize any soluble alkalis. Afterwards, rinse them once with tap water and again with distilled water.

(2) Soaking in detergent

After the pre-wash is finished, soak the glass instruments from a few hours to 24 h (depending on how dirty they are) in a solution of special synthetic detergent (Seven X, Hemozol, etc.) diluted to the concentration recommended by the manufacturer. If the instruments are very dirty, soak them in the aforementioned detergent for 20 to 30 min at a temperature of 70 to 80 °C, or soak them for 24 h in an alkaline detergent. These
detergents are appropriate for removing contaminants from the surface of glass, metal, plastic, ceramic, rubber, and other materials. Be sure, however, not to soak glass instruments with silicon oil or paraffin oil on them in the same sink with other instruments.

(3) Rinsing

After removing the instruments from the detergent, brush them if necessary; then place them immediately in a clean container with running water to rinse off the detergent. Rinse them with tap water on the inside and outside at least 10 times. Next, rinse them at least twice in distilled water, and at least twice again in redistilled water. If a glass container (except for those with a silicon coating) repels water, it has not been washed clean enough, and must be re-washed from the detergent soaking stage.

(4) Drying

Be careful that dust and other contaminants do not adhere to instruments while they are being dried. After the instruments are dried, if filmy streaks remain on them it means they are not clean enough, and they must be re-washed from the detergent soaking stage.

(5) Packing the instruments

After the instruments are washed and dried, they must be packed using aseptic methods appropriate for each type of instrument. For example, the mouths of glass instruments should be covered by two layers of aluminum foil. Plastic and metal instruments should be placed in special sterilization bags for the purpose of gas sterilization.

(6) Sterilization

The purpose of sterilization is to kill all microbes, whether pathogenic or not, and to prevent their reappearance. Described below are the principal sterilization methods for instruments used in embryo transfer procedures.

a) Dry heat sterilization

Dry heat sterilization achieves sterilization through the combined effects of temperature and time (either 160 °C for 90 min, or 180 °C for 60 min). Because dry heat conducts slowly and at uneven rates, the instruments must be arranged carefully in the sterilization chamber. This method is appropriate for glass instruments, which are resistant to high heat. The use of dry heat on weighing instruments can cause them to malfunction, so either moist heat sterilization or ethylene oxide gas sterilization should be used for these instruments.

b) Autoclave sterilization

Autoclaving achieves sterilization through the combined effects of temperature, pressure, and time. Almost all microbes are killed by applying vapor pressure of 1 kg/cm² (at 121 °C) for 5 min, but it is necessary to continue for 30 min to ensure complete sterilization. This method is appropriate for sterilization of glass instruments,
distilled water held in glass containers, solutions, culture media, paraffin oil, the tips of autopipettes, rubber stoppers, cloths, etc. Fully automatic autoclaves have recently become commercially available. Except for liquid solutions, the autoclave sterilization process includes sterilization, ventilation, and drying. Liquid solutions are only sterilized, after which the autoclave is allowed to air out slowly. When sterilizing a liquid solution, loosen its cap so that pressurized vapor can get inside the container. After the completion of sterilization, tighten the cap again securely. The water placed in the chamber should be distilled water.

c) Ethylene oxide gas (EOG) sterilization

The boiling point of EOG is 10.8 °C, so sterilization is carried out between normal room temperature and 60 °C. Because this method of sterilization can be carried out at lower temperatures than dry heat sterilization or autoclave sterilization, the instruments are almost never damaged by heat. EOG sterilization is appropriate for anything other than liquids, and is especially suited to the sterilization of balloon catheters made of rubber. With this method, sterilization is achieved through the combined effects of EOG concentration, temperature, humidity, and time. In order to completely remove all EOG from the instruments after sterilization is finished, the use of a heating chamber (60 °C) installed with a ventilation device is recommended. Plastic and rubber instruments, in particular, must be allowed to sit for at least a week before they are used so that EOG which has permeated them can escape (until EOG falls below a concentration of 100 ppm). EOG should not be used to sterilize products made of polyvinyl chloride plastic which have previously been sterilized by irradiation. EOG is harmful to human health, so be sure to place the sterilization equipment in a well ventilated room. It is also inflammable and heavier than air, so it must be handled with care. There are many types of EOG sterilization devices, but for the reasons stated above, a chamber is most effective.

Cited reference

IX. Examination of Embryos

Before embryos are transferred to a recipient, their developmental stage and morphology must be examined to determine whether they are likely to result in pregnancy, and what the pregnancy rate may be. In embryo transfer experiments, the assessment of embryo quality is a very important procedure to optimize pregnancy rates. The assessment of porcine embryo quality is based on standards developed for cattle. In the case of pigs, large numbers of embryos are transferred at one time, so it is difficult to determine the correlation between individual embryos and the overall pregnancy rate. It is best to complete the embryo examination procedure quickly, so skill is particularly important in the handling of pig embryos, since a large number of embryos must be examined.

At the current time, it is very common to use a microscope to examine the condition of embryos, and to assess their quality based on a certain criteria. This method requires experience, and the assessment tends to be subjective, thus there is a need to develop an objective means of assessing embryo quality.

<Required equipment and reagents>

<table>
<thead>
<tr>
<th>Product name</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stereoscope</td>
<td>Use at maximum magnification (40x - 50x) for quality assessment.</td>
</tr>
<tr>
<td>Inverted microscope</td>
<td>For quality assessment.</td>
</tr>
<tr>
<td>Microscope photography equipment</td>
<td>For photographing the condition of embryos.</td>
</tr>
<tr>
<td>Warming plate for microscope</td>
<td>Used to keep embryos warm while they are under the microscope.</td>
</tr>
<tr>
<td>Warming plate</td>
<td>Used to keep embryos warm while they are on the lab bench.</td>
</tr>
<tr>
<td>Clean bench</td>
<td>For preparing culture medium.</td>
</tr>
<tr>
<td>CO₂ incubator</td>
<td>Used as culture medium.</td>
</tr>
<tr>
<td>Culture medium</td>
<td>Used as culture medium.</td>
</tr>
<tr>
<td>Sterilization filter</td>
<td>For sterilization of culture medium through pressurized filtration.</td>
</tr>
<tr>
<td>10 ml syringe</td>
<td>For filter sterilization of liquid solutions.</td>
</tr>
<tr>
<td>Embryo manipulation pipettes</td>
<td>For washing embryos. Made from a Pasteur pipette.</td>
</tr>
<tr>
<td>Multiwell plate</td>
<td>For culturing of embryos.</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>To prevent evaporation of culture medium.</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>Kept in wash bottle, used to wash lab table, instruments, etc.</td>
</tr>
<tr>
<td>Record book</td>
<td>For keeping records.</td>
</tr>
</tbody>
</table>

1. Precautions regarding embryo examination

(1) Perform examination quickly so that the culture medium does not undergo a drastic change in temperature or pH, and does not evaporate.

(2) Be careful to keep instruments and culture medium free of bacteria.
Examinations are usually performed under a stereoscope, but an inverted microscope is sometimes used when examination and photography must be carried out at magnifications in the range of 100× to 200×. Be sure to record observations for each embryo.

2. Development of pig embryos

The stages of pig embryo development, and their respective characteristics, are as described below in Table IX-1.

<table>
<thead>
<tr>
<th>No. of days old</th>
<th>Stage of development</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-cell embryo</td>
<td>Must be distinguished from an unfertilized oocyte. Cytoplasm contains a large quantity of lipid droplets, which give it a dark appearance. If two polar bodies can be found, it is definitely fertilized.</td>
</tr>
<tr>
<td>2</td>
<td>2-cell embryo</td>
<td>Evenly divided.</td>
</tr>
<tr>
<td>3</td>
<td>4 to 8-cell embryo</td>
<td>Evenly divided. Embryos descend to uterus at this time.</td>
</tr>
<tr>
<td>4</td>
<td>Compact morula</td>
<td>In pig embryos, compaction occurs at the 4- to 8-cell stage. This leads to the early blastocyst stage.</td>
</tr>
<tr>
<td>5</td>
<td>Blastocyst</td>
<td>During the period from the initial formation of the blastocoele cavity until the vacuole accounts for half the volume of the embryo, the blastocyst is referred to as an early blastocyst. From this point until the cells fill the whole perivitelline space, it is called a blastocyst.</td>
</tr>
<tr>
<td>6</td>
<td>Expanded blastocyst</td>
<td>In this stage, the blastocoele expands and the zona pellucida decreases in thickness. The embryo sometimes hatches from the zona pellucida as early as this stage.</td>
</tr>
<tr>
<td>7</td>
<td>Hatched blastocyst</td>
<td>This stage begins after the embryo has hatched from the zona pellucida. Embryos which have reached this stage cannot be used for embryo transfer for preventing the spread of disease.</td>
</tr>
</tbody>
</table>
3. Assessment classification

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. (EXCELLENT)</td>
<td>No abnormality in either the shape of the embryo or of any individual cell. Normal development.</td>
</tr>
<tr>
<td>B. (GOOD)</td>
<td>10-20% of cells are protruding or degenerated.</td>
</tr>
<tr>
<td>C. (FAIR)</td>
<td>30-50% of cells are protruding or degenerated.</td>
</tr>
<tr>
<td>D. (POOR)</td>
<td>Embryo is misshapen, development lags, over 50% of the cells are abnormal.</td>
</tr>
<tr>
<td>E. (Infertility, fragmentation, etc.)</td>
<td>Unfertilized ova, degenerated ova, zona pellucida without vitelline contents etc.</td>
</tr>
</tbody>
</table>

4. Check points for morphological abnormalities

(1) Overall morphology and rate of development of the embryo

On rare occasions, an oocyte or embryo is collected which is thought to have been abnormal from the time of ovulation. There is a high probability that any embryo lagging one day or more behind the standard level of development is either an abnormal or low-ranking embryo.

(2) Morphology, thickness, damage to zona pellucida, and the presence/absence of sperm in the zona pellucida

On rare occasions, the zona pellucida has a protruding shape, but as long as the embryo is normal, this is not a problem. If the zona pellucida has cracks or other such damage, it may not be appropriate for use in embryo transfer for preventing the spread of disease. The size and thickness of the zona pellucida are criteria for determining whether the expanded blastocyst has shrunk or not. More sperm usually adhere to or penetrate the zona pellucida in pigs than in cattle or other farm animals, so if no sperm are found, the probability is very high that the oocyte is not fertilized.

(3) Shape, size, and darkness of each cell

The more uniform the size and darkness of individual cells, the higher the embryo is ranked. In the case of a 1-cell oocyte/embryo, check the two polar bodies that have been released to determine whether the oocyte has been fertilized or not. For embryos which have already developed to the expanded blastocyst stage or beyond, be especially careful to note whether the inner cell mass is clearly visible, as well as its shape and darkness.

(4) Percentage of protruding cells separated from the inner cell mass; degenerated cells with granulation; vacuolation

The lower these percentages, the higher the embryo is ranked. Embryos in the morula stage or beyond which have blastomeres in a protruding state (protruding cells,
generally in the process of degeneration and regression), or are degenerating through granulation or vacuolation, are ranked low.

Photo IX-1. 1-cell fertilized oocytes (1 day old)  
Photo IX-2. 2-cell embryo (2 days old)  
Photo IX-3. 4- to 8-cell embryos (3 days old)  
Photo IX-4. Compact morulae (4 days old); some have already begun to form blastocoele cavity.
Photo IX-5. Blastocysts (5 days old)

Photo IX-6. Expanded & hatched blastocysts (6 days old)

Photo IX-7. Expanded & hatching blastocysts (6.5 days old)

Photo IX-8. Hatched blastocysts (7 days old)
IX. Examination of Embryos

Photo IX-9. Extending blastocyst (15 days old)

Photo IX-10. Abnormal cleavage (Collected on 6th day of the estrous cycle, but no sperm were found in the zona pellucida. This condition is also referred to as fragmentation.)

Photo IX-11. Degenerated embryos

Cited reference
X. Embryo Transfer

With pigs, embryos are commonly transferred to the reproductive organs of recipient sows by means of a laparotomy operation with the sow under general anesthesia. This method is widely used because it offers consistently good results. In a surgical embryo transfer, the ovaries are exposed so that corpora lutea can be examined, and then the embryos are placed inside either an oviduct or uterine horn. With pigs, embryos migrate within the uterus, so all the embryos can be placed in either the left or right oviduct or uterine horn. Also, Appendix 1 includes a description of a non-invasive embryo transfer method in which the embryos are transferred to the recipient sow via the cervical canal.

<Required equipment and reagents>

<table>
<thead>
<tr>
<th>Product name</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine perforation needle (special order)</td>
<td>Only used when transferring embryos to uterine horn. As an alternative to using the uterine perforation needle, it is also acceptable to take a 1.2 mm x 10 cm (one end sharp, one end blunt) hypodermic needle of the same thickness, remove its handle, blunt the tip, and use it instead. It should fit snugly into a 0.25 ml straw.</td>
</tr>
<tr>
<td>0.25 ml straw</td>
<td>Cut off a 4 cm section to serve as a sheath for the perforation needle. If it is cut at an oblique angle, the catheter will insert more easily. The end that is inserted into the uterine horn should not have rough edges. Use gas to sterilize the perforation needle and straw together as a set.</td>
</tr>
<tr>
<td>1 ml syringe</td>
<td>Attached to the PP catheter.</td>
</tr>
<tr>
<td>PP catheter</td>
<td>Embryo transfer catheter.</td>
</tr>
<tr>
<td>Stereoscope</td>
<td>For examination of embryos.</td>
</tr>
<tr>
<td>Clean bench</td>
<td>For preparation of culture medium.</td>
</tr>
<tr>
<td>CO₂ incubator</td>
<td>For preservation of culture medium.</td>
</tr>
<tr>
<td>Culture medium</td>
<td>For preservation of culture medium. Also used as embryo transfer solution.</td>
</tr>
<tr>
<td>Sterilization filler</td>
<td>For sterilization of culture medium through pressurized filtration.</td>
</tr>
<tr>
<td>10 ml syringe</td>
<td>For filter sterilization of liquid solutions.</td>
</tr>
<tr>
<td>Embryo manipulation pipette</td>
<td>For washing embryos. Made from a Pasteur pipette.</td>
</tr>
<tr>
<td>Multiwell plate</td>
<td>For culturing of embryos.</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>Placed over culture medium to prevent evaporation.</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>Kept in wash bottle, used to wash lab table, instruments, etc.</td>
</tr>
<tr>
<td>Record book</td>
<td>For keeping records.</td>
</tr>
</tbody>
</table>

1. Transfer procedure
(1) Examination of ovaries <surgeon>

Examine both ovaries to be sure there are no abnormalities. While examining the ovaries, pour Chondron over them, and do not grip them with force. If the number of normal corpora lutea (generally five or more per ovary after hormone treatment) is appropriate given the timing of the examination, then there is no problem, but if there is a number of large follicles, then it is best not to use this animal as a recipient. After the
assistant has recorded the number of corpora lutea, leave only the part needed for the embryo transfer exposed (either an oviduct or an upper uterine horn), and return the rest of the reproductive organ to the abdominal cavity. Until the embryos to be transferred are ready, cover the exposed reproductive organs with gauze moistened with physiological saline containing Chondron.

(2) Preparing the embryos <assistant>

While monitoring the progress of the surgeon's preparations, prepare the embryos that are to be transferred. If the PP catheter is attached to a 1 ml syringe, its suction can be used to manipulate the embryos in the same manner as a pipette. After sufficient transfer fluid has been drawn in to leave the catheter half full, suction in a small amount of air followed by the embryos along with a small amount of fluid. Then suction in a small amount of air, followed by a small amount of fluid. The catheter is now prepared. Do not begin this step until the surgeon's preparations are completed. Do not touch the PP catheter directly with the hands, etc.

(3) Transfer to an oviduct

Be careful not to grip the oviduct with force. Introduce the PP catheter with the embryos via the infundibulum and insert the tip until it reaches the ampulla of the oviduct. Expel the embryos gently and remove the catheter. (While the catheter is being inserted into the oviduct by the surgeon, the 1 ml syringe connected to the PP catheter should be held by the assistant.)

(4) Transfer to a uterine horn [See Illustrations on next page, and Photo X-1 on page 79.]

① Using the sharp end of the perforation needle (to which a straw has been attached as a sheath), gently pierce a hole in the upper uterine horn in a location with relatively few blood vessels (ca. 5 to 10 cm from the place where the uterus and oviduct are joined together). Then, using the blunt end, insert the perforation needle into the uterine cavity. If bleeding occurs, suppress it lightly with gauze, etc.

② Leaving the straw in place, remove the perforation needle. The purpose of the straw is to keep a path open to the uterine cavity.

③ The PP catheter holding the embryos is now placed inside the straw, and inserted through the straw deeply into the uterine horn. The result is that the embryos are actually transferred to a location slightly removed from the spot where the uterine horn has been perforated. When the catheter is being inserted, the surgeon should hold the straw and the uterine horn in a position that makes it easy for the assistant to insert the catheter. In particular, the uterine horn should be held in a straight position so that the catheter will enter deeply and smoothly.

④ The assistant slowly pulls out on the catheter while expelling the fluid containing the embryos from the syringe. After all the liquid has been expelled, gently remove the catheter and straw. There is no special need to suture the spot where the uterine
horn was perforated. (The PP catheter with the 1 mm syringe attached to it is held by the assistant; the surgeon must be careful not to touch the straw or syringe.)

1. Using the sharp end of the perforation needle, gently pierce the surface of the uterine horn.

![Diagram](image)

2. With a cut off section of straw fitted over it as a sheath, insert the blunt end of the needle into the uterine cavity.

![Diagram](image)

3. Withdraw the perforation needle, leaving the straw in place.

4. Insert PP catheter holding the embryos through the straw and deeply into the uterine cavity.

![Diagram](image)

5. While slowly pulling out on the catheter, expel the embryos. After all embryos have been expelled, remove the straw and catheter.
X. Embryo Transfer

PP catheter
1 ml syringe

Small amount | Small amount | Approx. half the catheter length

Transfer fluid | Air | Transfer fluid | Air with embryos | Transfer fluid

Figure X-1. Embryo arrangement in the PP catheter

Photo X-1. Alternative method (using perforation needle to pierce hole in tip of uterine horn in spot with few blood vessels)

Photo X-2. Pasteur pipette holding embryos is inserted into the hole pierced as shown in photo above.
XI. Embryo Preservation

Embryos must be preserved or stored until they are transferred or used in an experiment, and preservation must not impair their ability to develop into piglets. In this chapter, we shall describe short-term preservation, embryo culture, and cryopreservation methods.

Neither the embryo culture method nor any other short-term preservation method has yet been perfected, so it is very difficult to completely avoid impairing the developmental capacity of preserved embryos. For this reason, it is best to keep the storage time as short as possible when these methods are used. Embryo preservation involves the handling of embryos outside the sow’s body, which requires caution. See Chapter VIII, Handling of Embryos, for information on proper handling procedures. In addition, only limited success has been achieved so far with cryopreservation techniques. Development capacity for thawed embryos is very low; the only embryos with tolerance to freezing are blastocysts collected around the time they are hatching from the zona pellucida. Further research and development are required in this area.

<Required equipment and reagents>

<table>
<thead>
<tr>
<th>Product name</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preservation medium</td>
<td>For embryo preservation.</td>
</tr>
<tr>
<td>Mill-Q water or deionized water that has been distilled twice</td>
<td>For preparation of preservation medium.</td>
</tr>
<tr>
<td>Top-plate counter scale</td>
<td>For weighing reagents.</td>
</tr>
<tr>
<td>pH meter</td>
<td>For measuring hydrogen ion concentration in the preservation medium.</td>
</tr>
<tr>
<td>Stirrer</td>
<td>For stirring during preparation of preservation medium.</td>
</tr>
<tr>
<td>Measuring flask</td>
<td>For preparation of preservation medium</td>
</tr>
<tr>
<td>Sterilization filter (diameter: 0.22 μm)</td>
<td>For sterilization of preservation medium using pressurized filter.</td>
</tr>
<tr>
<td>Syringe</td>
<td>For sterilization with filter.</td>
</tr>
<tr>
<td>Clean bench</td>
<td>Used during preparation of preservation medium.</td>
</tr>
<tr>
<td>PBS powdered synthetic culture medium (Gibco, Cat.No. 21600-010; Nissui, product number 05913)</td>
<td>Preservation medium</td>
</tr>
<tr>
<td>MEM (Gibco, Cat.No. 41500-034)</td>
<td>Container for preserved embryos</td>
</tr>
<tr>
<td>Tube with sterilized cap</td>
<td>Placed over preservation medium to prevent evaporation.</td>
</tr>
<tr>
<td>Sterilized 35x10 mm dish</td>
<td>To keep embryos warm.</td>
</tr>
<tr>
<td>Mineral oil (liquid paraffin, silicon oil)</td>
<td>To search for embryos.</td>
</tr>
<tr>
<td>Embryo manipulation pipettes</td>
<td>For incubation of embryos.</td>
</tr>
<tr>
<td>Warming plate or incubator</td>
<td></td>
</tr>
<tr>
<td>Steroscope</td>
<td></td>
</tr>
<tr>
<td>Culture medium</td>
<td></td>
</tr>
<tr>
<td>CO₂ incubator</td>
<td></td>
</tr>
<tr>
<td>Item</td>
<td>Description</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Freezing solution</td>
<td>For cryopreservation of embryos.</td>
</tr>
<tr>
<td>Freezing solution diluted with glycerin</td>
<td>For dilution of frozen-thawed embryos.</td>
</tr>
<tr>
<td>Glycerin</td>
<td>Cryoprotectants for embryos.</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Used for dilution after thawing.</td>
</tr>
<tr>
<td>PB1</td>
<td>Basal medium for freezing solution.</td>
</tr>
<tr>
<td>Culture medium</td>
<td>For culturing of embryos prior to freezing.</td>
</tr>
<tr>
<td>0.25 ml straw for freezing of semen</td>
<td>Container for embryos during freezing.</td>
</tr>
<tr>
<td>Straw powder or polysealer</td>
<td>For sealing the straw.</td>
</tr>
<tr>
<td>Straw cutter</td>
<td>For cutting the straw.</td>
</tr>
<tr>
<td>Programmable freezer</td>
<td>For cooling the embryos.</td>
</tr>
<tr>
<td>Tweezers</td>
<td>Used during ice seeding.</td>
</tr>
<tr>
<td>Timer or stopwatch</td>
<td>For measuring embryo manipulation time.</td>
</tr>
<tr>
<td>Liquid nitrogen</td>
<td>Medium for super low temperature cryopreservation of embryos.</td>
</tr>
<tr>
<td>Liquid nitrogen tank</td>
<td>For super low temperature cryopreservation of embryos.</td>
</tr>
<tr>
<td>Ampoule cans</td>
<td>Container for straw holding embryos in low temperature cryopreservation.</td>
</tr>
<tr>
<td>Record book</td>
<td>For keeping records.</td>
</tr>
</tbody>
</table>

1. Short-term preservation of embryos

The term "short-term preservation of embryos" is used in this manual to refer to the preservation of embryos for a short time without using an incubator, and when a half-day or less elapsed between collection and transfer or use for experimental purposes.

(1) Short-term embryo preservation medium

Culture media with either a phosphate or HEPES buffer, which retain a proper pH balance even when exposed to the air, are used as the short-term embryo preservation medium. In this section we shall describe the preparation of two kinds of embryo preservation media, one based on PB1 (with phosphate buffer) and one based on MEM (with HEPES buffer). M-2, which is described in Chapter VI, Surgical Collection of Embryos, can also be used as a short-term preservation medium. Because these preservation media retain a proper pH balance even when exposed to the air, they must be kept in a tightly capped tube when placed in a CO₂ incubator. After the liquid has been sterilized, the generally accepted procedure is to pour it into tubes with caps, place the tubes on a warming plate, and then use it once it has warmed up. The preservation medium can also be kept for about two weeks after it is prepared. It must first be sterilized using a sterilization filter, and then placed in a refrigerator. The water used for preparing the preservation medium should be either a super-purified water, such as Milli-Q water, or deionized water that has been distilled twice. When the preservation medium is used for embryo transport, for manipulation under the microscope, or for cryopreservation, inactivated fetal calf serum (FCS) is often added in concentrations of 10% to 20%.
a) Preparation of PB1

PB1 is the same as Dulbecco's PBS, except that sodium pyruvate, glucose, calf serum albumin, and antibiotics have been added. The components of PB1 are listed in Table XI-1. First, weigh all components other than calcium chloride and magnesium chloride, pour them into a container of super-purified water, and stir thoroughly with the stirrer. (The amount of water in the container should be 70% of the amount of preservation medium required.) Next, pour the calcium chloride and magnesium chloride into a separate container of super-purified water (20% of the amount of preservation medium required), and stir thoroughly. (If the chemicals are not completely dissolved, the solution will appear cloudy.) Pour the solution of calcium chloride and magnesium chloride slowly into the previously prepared phosphate solution to mix them, and pour the resulting solution into a measuring flask, and mix it. After the two solutions are mixed together, add blood serum, or serum albumin or other proteins, and sterilize the mixture with a sterilization filter.

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>800.0</td>
</tr>
<tr>
<td>KCl</td>
<td>20.0</td>
</tr>
<tr>
<td>CaCl₂/2H₂O</td>
<td>13.2</td>
</tr>
<tr>
<td>MgCl₂/6H₂O</td>
<td>10.0</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>115.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>20.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>100.0</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>3.6</td>
</tr>
<tr>
<td>Penicillin G (sodium salt)</td>
<td>10,000 U</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>BSA (fraction V)</td>
<td>400.0 mg</td>
</tr>
</tbody>
</table>

b) Preparation of HEPES-MEM

HEPES-Minimum Essential Medium (MEM) is a synthetic culture medium that comes in powdered form and contains many vitamins and amino acids. First, measure out the required amount of MEM powdered synthetic medium. Then measure out the required amounts of sodium bicarbonate (NaHCO₃) 4 mM (0.336 g/l), HEPES (5.4663 g/l if it contains sodium salt; 5.004 g/l if it contains HEPES acid), penicillin G, and streptomycin (see Table XI-2). Dissolve these components in the required amount of water and stir thoroughly with a stirrer. Next, measure the pH of this medium and add either 1N HCl or NaOH to adjust the pH to 7.3. Add the necessary amount of proteins and sterilize the medium with a sterilization filter.
Table XI-2. Components of Hapes-MEM for pig embryo preservation

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM synthetic powder*</td>
<td>9.6</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.336</td>
</tr>
<tr>
<td>HEPES** (Sodium salt)</td>
<td>5.4663</td>
</tr>
<tr>
<td>[Hepes acid]</td>
<td>5.004</td>
</tr>
<tr>
<td>Penicillin G (sodium salt)</td>
<td>10,000U</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>BSA (fraction V)</td>
<td>5 mg/ml</td>
</tr>
</tbody>
</table>

*: Minimum Essential Medium (Gibco BRL, Cat. No. 41500-034)
:**: Either Hapes acid or sodium salt is used.

(2) Container for keeping embryos warm

Dishes, tubes, and straws are usually used as embryo containers. Along with the medium containing the embryos, these are kept for the purpose of preservation at a temperature near that of the pig’s body temperature (37.0-39.0°C). In most cases, a pipette is used to place the embryos in a 10 x 35 mm dish containing 2 to 3 ml of preservation medium warmed in advance to 37.0-39.0°C, and the embryos are kept there for several hours until the transfer procedure is performed in the laboratory or elsewhere. After the embryos have been placed in the dish, a warming plate set to 38°C is placed over the dish. Preservation medium evaporates when this method is used, so if the embryos must be preserved for a relatively long period, the microdroplet culture method is used. This method is outlined below.

### Microdroplet culture method

1. In a 35x100 mm dish, a pipette (with sterilized tube attached) is used to prepare 30-100 μl drops of culture medium (preservation medium).
2. Using a sterilized pipette, transfer 3.0-3.5 ml of mineral oil (silicon oil and liquid paraffin are also acceptable) to the dish described in step 1.
3. Using the pipette, transfer embryos to the drops of preservation medium and keep them warm. (Place them in an incubator and leave them undisturbed.)

This method offers two principal advantages; contamination by microbes is unlikely to occur, and it is easy to observe and handle the embryos. It is necessary to verify beforehand that the mineral oil, silicon oil, or liquid paraffin to be used is not toxic to embryos. Pour a small amount of preservation medium into a dish. To prevent evaporation and contamination by microbes, it is sufficient to cover the surface of the medium with one of these oils. Place the embryos inside a capped tube (ca. 10 ml)
containing an appropriate amount of preservation medium. The tube can be kept warm by placing it in a temperature-controlled water bath, etc. When the embryos are to be transported, place them along with the preservation medium into a tube or straw, and keep them warm during transport by placing them in a vacuum bottle or a cell culture transport device (Fujihira Industries). Inactivated fetal calf serum (FCS) is often added to the preservation medium in concentrations of 10% to 20%.

(3) Short-term preservation at 20°C

If the procedures described above are used for short-term preservation of pig embryos (4-cell through blastocyst stages) at 20°C, embryo development can be suspended or slowed down. When embryos at a certain stage of development are required for an experiment, or when the estrous cycle of an embryo recipient is far behind that of the embryo donor, this method can be used to keep the embryos at a desired stage. However, the capacity of embryos to develop into piglets may be seriously impaired if this method is used to preserve the embryos for 48 h. Preservation time should be limited to 24 h or less.

2. Culturing of embryos

Among the methods available today for preserving embryos outside the sow’s reproductive tract, embryo or in vitro culture is the best way to maintain the developmental capacity of the embryos. Even with incubation, however, the longer the preservation time, the more impaired the developmental capacity becomes. There are three types of embryo culture methods: organ culturing, co-culturing with epithelial cells from the oviduct, and culturing in a defined medium. In this section we shall describe the microdroplet culture method, which is the most common method for culturing in a defined medium. Excellent review articles regarding the culture of pig embryos have been published (Davis, 1985; Petters and Wells, 1993). This is highly recommended reading.

1. Prepare the culture medium.
2. In a dish, prepare 30-100 μl drops of culture medium (required as preparation for the microdroplet culture method).
3. Place the dish prepared in step 2 in a CO₂ incubator to achieve equilibrium of the gases.
4. Transfer embryos to the drops of preservation medium.
5. With the embryos in the dish, culture them in the CO₂ incubator.

(1) Culture medium

The nutritional requirements of pig embryos depend on their stage of development. From the 1-cell stage to the compact morula stage, they can develop in a
simple salt solution. From the blastocyst stage to the hatched blastocyst stage, however, serum, amino acids, and other substances play an important role in embryo development, so nutritional requirements are higher once embryos reach the blastocyst stage. Most pig embryo culture media that have been described in the literature so far were originally developed for culturing the embryos of mice and other laboratory animals (see Table XII-3). Different groups report slightly different results in the culture of pig embryos, so it is best to compare several different culture media before beginning to culture pig embryos. An osmotic pressure of approximately 255 mOsm is reported to be appropriate, which is slightly lower than in culture media used for mice, sheep, and cattle. For culture media which contain a bicarbonate buffer, the dish should not be removed from the incubator while embryos are being cultured or, if removed it should be returned as quickly as possible. Furthermore, at least 2 h before embryos are cultured, they should be placed in a CO₂ incubator to achieve equilibrium of the gases.

a) Preparation of the culture medium

The water used for preparing the culture medium should be either a super-purified water, such as Milli-Q water, or deionized water that has been distilled twice. First, while verifying the contents of the culture medium, weigh all components other than calcium chloride and magnesium chloride. Pour them into a beaker of water, and stir it thoroughly with the stirrer. (The amount of water in the container should be 70% of the amount of preservation medium required.) Next, pour the calcium chloride and magnesium chloride into a separate container of water (20% of the amount of preservation medium required), and stir thoroughly. (If the chemicals are not completely dissolved, the solution will appear cloudy.) Pour the solution of calcium chloride and magnesium chloride slowly into the other solution to mix them. After the two solutions are mixed together, pour the resulting solution into a measuring flask, and add enough water to make up the required volume of culture medium. If the medium contains HEPES buffer, add either 1N HCl or NaOH to adjust the pH to 7.3. After the medium has been prepared, add the necessary amount of serum, serum albumin or other proteins, and sterilize it with a sterilization filter. Passing the culture medium through a pressurized sterilization filter will raise the pH level by about 0.1. In addition, be aware that there are two types of HEPES, Heps acid (FW = 238.3) and sodium salt (FW = 260.3). This must be taken into account when the components are weighed. After the culture medium has been sterilized, it can be preserved for a period of time if it is placed in a refrigerator. However, glutamine remains usable for only one week, so culture media containing glutamine should be prepared just before they are to be used.

b) Culturing conditions

Embryos are usually cultured at 38.5°C (37.0-39.0°C), 100% humidity, and with a gas mixture of 5% CO₂, 5% O₂, and 90% N₂ (5% CO₂ in air is also acceptable). If a CO₂ incubator is not available, the embryos can be cultured under normal atmospheric
conditions as long as part of the sodium bicarbonate in the culture medium listed in Tables XII-3 and XII-4 is replaced with HEPES.

Table XII-3. Components in the most commonly used pig embryo culture media (Part 1)

<table>
<thead>
<tr>
<th>Component</th>
<th>Whitten's medium</th>
<th>Whitten's medium</th>
<th>Whitten's medium</th>
<th>Whitten's medium</th>
<th>BMOC-2</th>
<th>M-KRB</th>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>68.49</td>
<td>68.49</td>
<td>68.49</td>
<td>68.49</td>
<td>94.88</td>
<td>94.88</td>
</tr>
<tr>
<td>KCl</td>
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<td>4.78</td>
<td>4.78</td>
<td>4.78</td>
<td>4.78</td>
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<td>CaCl₂</td>
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<td>---</td>
<td>---</td>
<td>1.71</td>
<td>1.71</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19</td>
</tr>
<tr>
<td>MgSO₄ • 7H₂O</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25.07</td>
<td>25.07</td>
<td>25.07</td>
<td>25.07</td>
<td>25.07</td>
<td>25.07</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.56</td>
<td>5.56</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>5.56</td>
</tr>
<tr>
<td>Glutamine</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Taurine</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Hypotaurine</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Sodium lactate</td>
<td>25.20</td>
<td>25.20</td>
<td>25.40</td>
<td>12.93</td>
<td>25.00</td>
<td>29.20</td>
</tr>
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<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Calcium (lactate)₂</td>
<td>1.71</td>
<td>1.71</td>
<td>1.71</td>
<td>1.61</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>EDTA</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Na₂HPO₄ • H₂O</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MgCl₂ • 6H₂O</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>HEPES</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>BSA (mg ml⁻¹)</td>
<td>4.00</td>
<td>15.00</td>
<td>4.00</td>
<td>4.00</td>
<td>1.00</td>
<td>4.00</td>
</tr>
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</table>

Discussed in reference(s) #...

1  2, 3, 4  5  6  7  8
# XI. Embryo Preservation

Table XII-4. Components in the most commonly used pig embryo culture media (Part 2)

<table>
<thead>
<tr>
<th>Component</th>
<th>NCSU 6</th>
<th>NCSU 23</th>
<th>NCSU 37</th>
<th>TLH</th>
<th>CZB</th>
<th>H-CZB</th>
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<tr>
<td>NaCl</td>
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<td>4.83</td>
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<td>2.00</td>
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<td>KH₂PO₄</td>
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<td>1.19</td>
<td>...</td>
<td>1.18</td>
<td>1.18</td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19</td>
<td>...</td>
<td>1.18</td>
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<td>NaHCO₃</td>
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<td>25.07</td>
<td>25.07</td>
<td>2.00</td>
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<td>4.00</td>
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<tr>
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<td>5.55</td>
<td>5.55</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Glutamine</td>
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<td>1.00</td>
<td>1.00</td>
<td>...</td>
<td>1.00</td>
<td>...</td>
</tr>
<tr>
<td>Taurine</td>
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<td>7.00</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Hypotaurine</td>
<td>...</td>
<td>5.00</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Sorbitol</td>
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<td>...</td>
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<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Sodium lactate</td>
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<td>...</td>
<td>10.00</td>
<td>31.30</td>
<td>31.30</td>
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<tr>
<td>Sodium pyruvate</td>
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<td>...</td>
<td>0.25</td>
<td>0.27</td>
<td>0.27</td>
</tr>
<tr>
<td>Calcium (lactate)₂</td>
<td>...</td>
<td>...</td>
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<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
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<td>...</td>
<td>0.11</td>
<td>0.11</td>
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<tr>
<td>Na₂HPO₄ · H₂O</td>
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<td>...</td>
<td>0.40</td>
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<td>...</td>
</tr>
<tr>
<td>MgCl₂ · 6H₂O</td>
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<td>...</td>
<td>...</td>
<td>0.50</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>HEPES</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>10.00</td>
<td>...</td>
<td>20.00</td>
</tr>
<tr>
<td>BSA (mg ml⁻¹)</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>6.00</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Phenol red (mg ml⁻¹)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>0.01</td>
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</tbody>
</table>

Discussed in reference(s) #... 9 10 11 12 13 14
3. Deep freezing method

The deep freezing method is extremely important in the effort to achieve more widespread use of embryo transfer techniques. The deep freezing method is a valuable resource in the field of biotechnology because it can be used for semi-permanent preservation of genes in the form of complete genomes. It is also valuable because it can be used for preservation during embryo transport. Furthermore, as genetic engineering becomes more sophisticated, it is likely that the deep freezing method will become increasingly important as a means of preserving transgenic animals. With the current deep freezing method, however, the developmental capacity of frozen-thawed pig embryos is very low, and the only embryos with tolerance to freezing are blastocysts collected around the time they are hatching from the zona pellucida. The deep freezing method has not yet been perfected. Recent research has shown that the tolerance of pig embryos to freezing is closely related to the presence of lipid droplets in the embryo cells; thus, there is considerable hope that this discovery will lead to further technical advances. According to current research, when cleaved embryos are centrifuged to concentrate the lipid droplets and these droplets are then removed with a micropipette, the embryos become more tolerant to freezing, and are able to develop into normal offspring after thawing and transfer. In this section, we shall describe the most commonly used deep freezing method. With this method, 1.5 M glycerin is used as the cryoprotectant, and the embryos are cooled in a programmable freezer.

(1) Freezing method

1. Select embryos.
2. Fill a straw with freezing solution.
3. Set the programmable freezer’s chamber temperature to cool to -6.5°C.
4. Place the embryos in a freezing solution containing 1.5 M glycerin.
5. Transfer the embryos to a straw and seal the straw.
6. Place the straw in the chamber of the programmable freezer.
7. Using tweezers (or a similar tool) cooled in liquid nitrogen (LN$_2$), carry out ice seeding.
8. Set the programmable freezer to cool the embryos at 0.3 - 0.5°C/min. until they reach a temperature of -30°C.
9. Put the embryos in the liquid nitrogen.
10. Store the embryos in the liquid nitrogen.
(2) Thawing method

1. Remove the straw from the liquid nitrogen and expose it to the air for about five seconds.
2. Put the straw in warm water (20-30°C) and shake it for about ten seconds.
3. Perform a three-step dilution procedure to remove the glycerin.
4. Wash three times in culture medium.
5. Transfer the embryos to either a culture medium or a recipient sow.

(3) Preparation of medium for freezing/dilution

The basal culture medium used for freezing and diluting embryos is usually prepared by adding inactivated FCS or calf serum albumin to either Dulbecco's PBS (see Table VI-2) or PB1 (see Table XI-1). In this section, we shall describe the preparation of a freezing solution containing 1.5 M glycerin or 0.25 M sucrose, as well as the preparation of a stepwise dilution medium for removing glycerin from thawed embryos. First, prepare a 3.0 M glycerin solution and a 1.0 M sucrose solution to serve as stock solutions (the basal medium acts as a solvent). To make 9.5 ml of 3.0 M glycerin solution, mix 2.1 ml of glycerin with 7.4 ml of basal medium. By mixing these stock solutions together with the basal medium in the proportions described in Table XI-5 (next page), it is easy to prepare the freezing solution and stepwise dilution medium. For example, to make 4 ml of freezing solution containing 1.5 M glycerin and 0.25 M sucrose, mix together 2 ml of 3.0 M glycerin solution, 1 ml of 1.0 M sucrose solution, and 1 ml of basal medium. These stock solutions can be preserved for several months when stored frozen at -20°C.

(4) Embryo selection

The tolerance of pig embryos to freezing varies greatly depending on the stage of development. Embryos with the best tolerance to freezing are those which are soon to or have already hatched from the zona pellucida (especially those which have just hatched), have an outer diameter of 300 μm or less, and are still light-colored (have not yet turned dark). Proper embryo selection is important to the survival rate of thawed embryos.
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Table XI-5. Preparation of 1.5 M glycerin solution and stepwise dilution medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>Stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.0 MG</td>
</tr>
<tr>
<td>Freezing solution</td>
<td></td>
</tr>
<tr>
<td>1.5 MG</td>
<td>1.0</td>
</tr>
<tr>
<td>1.5 MG+0.25 MS</td>
<td>2.0</td>
</tr>
<tr>
<td>Stepwise dilution medium</td>
<td></td>
</tr>
<tr>
<td>1  0.75 MG+0.3 MS</td>
<td>1.0</td>
</tr>
<tr>
<td>2  0.30 MS</td>
<td>---</td>
</tr>
<tr>
<td>3  0.15 MS</td>
<td>---</td>
</tr>
</tbody>
</table>

G: Glycerin, S. Sucrose

(5) Taking embryos into the straw, ice seeding, and cooling

Wash the inside of a 0.25 ml straw two or three times using freezing solution. Be sure that the freezing solution does not come into contact with the cotton plug. Then draw the freezing solution into the straw in the manner shown in Figure XI-1.

![Figure XI-1. Arrangement of freezing solution in the straw](image)

First, set the programmable freezer's chamber temperature to cool to -6.5°C. Next, place the embryos (10-20 per straw) in a dish containing freezing solution at room temperature (ca. 20°C). Handle with care, as the embryos may float on the surface of the freezing solution. Using an embryo manipulation pipette, immediately place the embryos in the section of straw that holds the freezing solution (section labeled 1-FS in Figure XI-1), then seal the straw using either straw powder, polysealer, or the like. Beginning when the embryos are placed in the freezing solution, use a stopwatch or other such device to time the procedure. Five minutes after the embryos have been sealed inside the straw, place the straw in the programmable freezer, the chamber of which has been cooled to -6.5°C. Immediately after this is done, start the freezer's cooling program, which has been set beforehand (see Figure XI-2). Within 1 min after the straw containing the embryos is placed in the chamber at -6.5°C, carry out ice seeding. Seeding must be carried out quickly using a pair of tweezers cooled
beforehand in liquid nitrogen. These tweezers are used to grasp the section of straw where the embryos are located. The straw is then monitored to check for the growth of ice crystals. The straw is kept at -6.5°C for 10 min, and is then cooled to -30°C at 0.3°C/min. Once it reaches -30°C, it is kept at this temperature for 3 to 5 min. before being placed in liquid nitrogen (LN₂). This completes the freezing process. After the end of the 10 min during which the straw is kept at -6.5°C, it takes about 1.5 h for the embryos to cool to -30°C.

![Flowchart](image)

Figure XI-2. Pig embryo cooling program

(6) Thawing and glycerin removal through stepwise dilution

Use tweezers or a similar tool to remove the straw from the liquid nitrogen. To thaw the embryos, hold the straw in the air for ca. 5 sec, then place it for ca. 10 sec in warm water (20-30°C). Use a Kimwipe or similar cloth to wipe any water off the surface of the straw. With a straw cutter, snip off first the sealed part of the straw, then the plugged part, and empty the embryos from the straw into the dish along with the freezing solution. Next, place the thawed embryos in each of the stepwise dilution liquids (listed below) for 5 min apiece to remove the glycerin. Then wash the embryos three times in a solution of PB1 + 10% FCS. After the glycerin has been removed, place the thawed embryos in culture medium or transfer them to a recipient sow. The probability of survival for thawed embryos can be determined by examining their shape after they have been cultured.
4. Successful cases of pig embryo cryopreservation

At the time this manual was written and edited, pig embryo cryopreservation had already been applied with success in several cases. Reported cases are listed in Table XI-6 below. Embryos have also been successfully preserved through vitrification, although these are not listed in Table XI-6. For further information on vitrification, see Appendix 3.

Table XI-6. Successful cases of pig embryo cryopreservation: production of piglets from frozen-thawed embryos

<table>
<thead>
<tr>
<th>Reported by</th>
<th>Year</th>
<th>Cryoprotectant</th>
<th>Embryos transferred</th>
<th>Piglets produced</th>
<th>Survival rate (%)</th>
<th>Discussed in reference</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Oguri et al</td>
<td>1989</td>
<td>1.4 MG+egg yolk</td>
<td>8</td>
<td>1</td>
<td>12.5</td>
<td>16,17</td>
<td></td>
</tr>
<tr>
<td>Kameyama et al</td>
<td>1990</td>
<td>1.5 MG+lecithin</td>
<td>20</td>
<td>4</td>
<td>20.0</td>
<td>18</td>
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<tr>
<td>Kashiwazaki et al</td>
<td>1991</td>
<td>1.5 MG</td>
<td>32</td>
<td>4</td>
<td>12.5</td>
<td>19,20,21</td>
<td></td>
</tr>
<tr>
<td>Nagashima et al</td>
<td>1995</td>
<td>1.5 MPROH+0.1 MS</td>
<td>39</td>
<td>3</td>
<td>7.7</td>
<td>22</td>
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<tr>
<td>Nagashima et al</td>
<td>1995</td>
<td>1.5 MG+0.25 MS</td>
<td>66</td>
<td>4</td>
<td>6.1</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

G: glycerol, PROH: 1,2-propanediol, S: sucrose
Cited references


Further suggested readings

- Kashiwazaki N. Culture of pig embryos The Eastern Japan Embryo Transfer Society 1991;7, 36-49.
- Nagashima H, Kashiwazaki N, Ashman RJ, Gruppen CG, Seamark RF, Nottle M. Recent advances in cryopreservation of porcine embryos. Theriogenology 1993; 41, 113-118.

XII. Anesthesia

An anesthetic is a drug which reversibly suppresses the function of nerve cells, but it is not fully understood how anesthesia works. Anesthesia can be broadly categorized into three types: local, general, and special anesthesia (acupuncture, electroacupuncture, etc.).

When general anesthesia is needed for surgery, ideally the four following conditions are achieved: analgesia, loss of consciousness, suppression of reflexes, and relaxation of muscles. The autonomic nervous system in pigs is very sensitive, and pigs will collapse when highly excited or subjected to sharp pain. The porcine airways are very narrow while the neck is thick, an anatomical feature which can cause difficulty of breathing. Some anesthetics can induce low blood pressure, a slow pulse, and malignant hyperthermia. In pigs weighing over 70 kg, it is reported that some anesthetics can be absorbed by the fat, making it difficult to achieve anesthesia and causing the pig to recover from anesthesia very slowly. For these reasons, there is a high degree of uncertainty and danger in the administration of general anesthesia to pigs. Laparotomy operations are essential for embryo collection, however, so there is a need to develop a safer method of anesthesia for this procedure.

A number of outstanding new anesthetics which offer a high degree of safety have been developed recently, but sensitivity and pharmacological action vary depending on the type of animal to which they are administered. We shall leave description of these new anesthetics to other more specialized publications. In this chapter, we shall concern ourselves with the description of anesthetics which are widely used for (or are expected to be effective in) laparotomy operations in pigs, and shall discuss their use in general anesthesia.

1. Anesthetics
   (1) Preanesthetic medication

When an animal is taken into an operating room, the unfamiliar environment and the busy comings and goings cause unease, fear, and tension, and sometimes trigger resistance on the part of the animal to being handled. This makes it difficult to achieve and maintain anesthesia, and the animal cannot be expected to remain quiet after recovering from anesthesia. For the safety of both the animal and humans, and for the sake of the animal's welfare, an effort must be made to keep the animal sedate at all times.

Before anesthesia is attempted, preanesthetic medication is administered so that anesthesia can be carried out safely and smoothly. Preanesthetic medications are classified into two categories. One category includes parasympathetic nervous system suppressants (anticholinergic drugs), which are effective in preventing an overly
stressed parasympathetic nervous system from causing excessive salivation and secretions in the airways, convulsions in the bronchial tubes and larynx, vomiting, loss of blood pressure, slow pulse, and cardiac arrest. The second category includes a number of drugs which have anticholinergic effects and also suppress the central nervous system. These medications prevent fear, excitement, and the sudden administration of anesthesia from subjecting the sympathetic nervous system to excessive stress; thus, they are useful in preventing hypermetabolism, tachycardia, cardiac arrhythmia, and ventricular fibrillation. Described below are some of the preanesthetic medications used with pigs.

A. Anticholinergic agents
   i. Atropine sulfate
      1. Suppresses mucous secretions by the airways, prevents expansion of the bronchial tubes and convulsions of the larynx, and has an anticholinergic effect.
      2. In pigs, atropine sulfate increases the viscosity of the mucus. It does not have a clear effect on bradycardia. It is now being used less due to the recent development of more effective new drugs.

B. Tranquilizers
   Tranquilizers do not have any analgesic effect.
   i. Phenothiazine derivatives
      Phenothiazine derivatives suppress the central nervous system, prevent vomiting, and induce sedation and lower blood pressure by acting as α-adrenoreceptor antagonists.
   a. Chlorpromazine hydrochloride (Chontomine)
      1. Prevents vomiting, lowers body temperature, relaxes muscles, reduces secretion of gastric juices, etc.
      2. Relaxes the small intestine, bladder, uterus, etc.
      3. Duration of effect of intramuscular injection varies slightly; 45 to 60 min required for tranquilization.

b. Acepromazine maleate
   Acepromazine maleate is widely used in veterinary practice in Europe and North America because of its low toxicity and powerful effect as a nervous system suppressant, but it is not commercially available in Japan. It is especially popular among small animal practitioners.
   1. Very effective tranquilizer. Suppresses the central nervous system, prevents vomiting, acts as a muscle relaxant, and suppresses voluntary movements.

ii. Butyrophenone derivatives
   Butyrophenone derivatives cause the animal to become less aware of its surroundings, and make its movements torpid, but have no analgesic effect. Have slight effect upon circulatory and respiratory organs. Have a very strong antiemetic effect.
   a. Azaperone (Stresnil®)
      1. Very effective tranquilizer.
      2. Administering azaperone and then ketamine is a very safe tranquilizing method.
C. Sedatives (sedative/hypnotics)

These drugs cause sedation and also have an analgesic effect.

i. $\alpha_2$-adrenoceptor agonists

$\alpha_2$-adrenoceptor agonists act as a nerve blockers.

a. Xylazine hydrochloride [Rompun®]

1. Acts as a sedative, hypnotic, analgesic, and muscle relaxant.
2. There are two types of antagonists for xylazine hydrochloride: yohimbine ($\alpha_2$-adrenoceptor antagonist) and doxapram.
3. In pigs, xylazine hydrochloride tends to stimulate the central nervous system, so it is safer to use it in combination with other drugs.
4. Cows and other ruminants are extremely sensitive to xylazine hydrochloride.

b. Medetomidine hydrochloride (Dormitrol)

1. Medetomidine hydrochloride is stronger than xylazine hydrochloride as both a sedative and a muscle relaxant.
2. Lowers body temperature.
3. The antagonist for medetomidine hydrochloride is atipamezole.

D. Analgesics

a. Butorphanol (Stadol or Torbugesic®)

1. Butorphanol is a non-narcotic analgesic.
2. Has little or no sedative effect.
3. Enhances the sedative effect of medetomidine hydrochloride.

(2) Intravenous injectable anesthetics

It takes a long time for an animal to wake up after administration of an intravenous injectable anesthetic, and it is difficult to adjust the depth of anesthesia. In the case of barbiturates (thiobarbiturates), the removal of anesthetic from the blood and brain are achieved principally by conversion in the liver and elimination by the kidneys along with urine. In order to administer intravenous injectable anesthetics safely and effectively, it is necessary to have an understanding of the pharmacological characteristics of the drug being used, to have the knowledge required to interpret how the animal reacts to the drug, and to have the skills needed to deal with all contingencies. Intravenous anesthetics can be broadly classified into the two categories described below.

A. Barbiturates

Barbiturates have little or no hypnotic, analgesic, or muscle relaxant effect. They are used as intravenous injectable anesthetics.

i. Oxybarbiturates

Oxybarbiturates have a short- to medium-term effect.

a. Sodium pentobarbital (Nembutal®, Somnopentyl®)

1. Has a hypnotic effect.
2. Has little analgesic or muscle relaxant effect.

There are two types of adrenoceptors: $\alpha_1$ and $\alpha_2$ receptors. When a sympathetic agent acts upon an $\alpha_1$ receptor, it causes constriction of blood vessels and slight contraction of the bronchial tubes. This is called the vasoconstrictor. There are two types of $\alpha_1$ receptors, $\alpha_1_a$ and $\alpha_1_b$. When a sympathetic agent acts upon an $\alpha_2$ receptor, it suppresses the release of noradrenaline (norepinephrine) from nerve endings (see previous page).
 Suppresses the vasomotor center and dilates peripheral blood vessels, which causes a slight drop in blood pressure.

4. Strong suppressant of the inspiratory center.

5. When liver function is deteriorated due to injury, sodium pentobarbital will cause further deterioration.

6. Sodium pentobarbital dilates the blood vessels in the spleen. In the peripheral blood, this lowers the red blood cell count, reduces the concentration of hemoglobin, and reduces hematocrit values.

7. In pigs, sodium pentobarbital frequently causes stoppage of spontaneous breathing.

8. If breathing stops, administer doxapram hydrochloride (5-10 mg/kg).

ii. Thiobarbiturates

Thiobarbiturates have a ultrashort-term effect.

a. Sodium thiopental (Labonaru) Pentothal® sodium as well as several other names.

1. Acts as a strong suppressant of the central nervous system (for 15 to 30 min), but has no analgesic effect.

2. Causes tachycardia, lowers blood pressure, suppresses myocardial contraction. Sometimes causes arrhythmia.


4. In light anesthesia, causes convulsions of the larynx and bronchial tubes.

5. Acts as muscle relaxant.

b. Sodium thiamylal [Surital®]

The pharmacological action of sodium thiamylal is roughly the same as that of sodium thiopental, but sodium thiamylal is a slightly stronger anesthetic.

B. Non-barbiturates

a. Ketamine hydrochloride [Ketalar®]

1. Can be administered orally or through subcutaneous or intramuscular injection.

2. Suppresses part of the central nervous system (hypothalamus, neocortex, subcortex), but does not cause complete loss of consciousness. Strong analgesic. Suppresses central breathing.

3. Increases heart rate and blood pressure.

4. Does not act as muscle relaxant.

5. None of the easily administered anesthetics that are not administered through intravenous injection are as dependable as ketamine hydrochloride.

b. Guaiacol glyceryl ether (GGE or guaifenesin)


2. Pharmacological actions include reduction of fever and inflammation.

(3) Inhalant anesthetics

Pigs are relatively insensitive to inhalant anesthetics. Even when a halothane inhaler is set to the maximum 4% concentration, it is impossible to be sure of satisfactory results. For general anesthesia, however, inhalants are safer than intravenous injection. There are two main categories of inhalant anesthetics, as described below.
XII. Anesthesia

A. Gas anesthetics

Gas anesthetics remain in the gaseous state at room temperature.

a. Nitrous oxide (also known as laughing gas)

1. A non-flammable, non-explosive gas. 1.5 times heavier than air. Kept compressed in liquid state in tank at pressure of 40 atmospheres. Changes safely to gas at 20°C.
2. Stable with respect to soda lime. Is not irritating.
3. Not irritating to airways. Does not suppress respiratory or myocardial function.
4. Causes contraction of the peripheral vessels and α-adrenoceptors. Inhale mixture of oxygen, halothane, and nitrous oxide is recommended to avoid causing low blood pressure.
5. Strong analgesic.
6. Does not act as a muscle relaxant.
7. Excreted from body without being metabolized.
8. Expensive.
9. Not used by itself, but in combination with the volatile anesthetics listed below in order to reduce strong side effects and allow lower dosages.

B. Volatile anesthetics

Volatile anesthetics remain in the liquid state at room temperature.

a. Halothane [Fluothane®]

1. Halothane is a hydrocarbon fluoride. Its specific gravity is 1.86, and its boiling point is 50.2°C.
2. Unlike ether, it is non-flammable and non-explosive.
3. Does not react to soda lime.
5. Respiratory and myocardial contraction suppressed in proportion to halothane concentration. Surgical stress causes shallow, rapid breathing.
6. Causes transient low blood pressure. In deep anesthesia, causes bradycardia (can be prevented with atropine).
7. Most halothane is eliminated through exhalation.
8. Tends to lower body temperature. On rare occasions, causes malignant hyperthermia.

b. Isoflurane [Forane®]

1. An isomer of enfurane, with a boiling point of 47-50°C.
2. A colorless, transparent liquid with a specific odor.
4. Stable with respect to soda lime, has little harmful effect upon liver and kidneys. Superior in this respect to halothane.

2. Administration of anesthesia

When carrying out general anesthesia, carefully note the various signs and physical changes that indicate depth of anesthesia. It is important not to suppress the central nervous system any more than necessary.

The signs and physical changes to be noted include the following:

---

The strength of inhalant anesthetics is expressed and determined in units called MACs (minimum alveolar concentrations). The concentration (%) required to elicit a reflex response from 50 percent of animals under anesthesia when subjected to painful stimulation is referred to as 1 MAC. The lower the MAC value, the stronger the anesthetic.
Stage I: Central nervous system becomes excited (locomotive center becomes excited, ability for coordinated movement is lost.)

Stage II: Contraction of all body muscles. Animal becomes delirious.
   A: Delirium
   B: Loss of consciousness. Events occurring during this stage are remembered when the animal recovers from anesthesia.
   C: Reaction to stimuli declines markedly. Righting reflexes are lost, and events occurring during this stage are not remembered.

Stage III: No response to stimuli. Regular, slow breathing. All central nervous system functions suppressed (surgical anesthesia stage). Stage III anesthesia is divided into three phases according to the depth of anesthetization (see Table XII-1).

Stage IV: Stage of medullary paralysis (toxic stage)
   Many anesthetics go from stage I into stage II, but any anesthetic which only goes as far as stage II B cannot be used (events that take place in stage II B are remembered afterwards). Halothane goes from stage I to stage III. Ether goes from stage II to stage III, but any animal which advances from stage I or II to stage III will die if it continues into stage IV. Use of ketamine is a relatively reliable means of attaining stage II C anesthesia even when it is administered using a method (such as intramuscular injection) which cannot be counted on to provide a particular concentration in the blood. It will not advance anesthesia to stage III, however, and if it continues to be administered after stage II C has already been attained, chronic muscle spasms will occur (i.e.,--muscles alternately relax and contract), which lead to convulsions throughout the entire body, respiratory failure, and death. Laughing gas only advances anesthesia from stage I to stage II C.

3. General anesthesia
   (1) Preparations
   A. Starvation and deprivation of water
      If there is much food in the stomach, it may be regurgitated and enter the trachea, so the animal should be starved beginning 12 h before anesthesia. For the same reason, the animal should be deprived of water beginning 2 to 3 h before anesthesia.

   B. Restraining the animal
      Use either a wire or a rope to restrain the snout (with the ring placed near the snout; the closer the ring is perpendicular to the upper jaw, the less likely the ring is to slip out). Pull strongly forward on the handle of the restrainer to make the animal stand up. When laying the animal on its side, either support it or use a revolving operating table to prevent the animal from being dropped.
Table XII-1. Features of stage III anesthesia

<table>
<thead>
<tr>
<th>Depth</th>
<th>Breathing</th>
<th>Animal’s physical condition</th>
<th>Muscle relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase II (medium anesthesia)</td>
<td>Regular, Chest and Abdominal.</td>
<td>Eyeball position is fixed. Corneal reflexes are lost.</td>
<td>Coughing continues until mid-stage through this phase. Almost all muscles (except abdominal muscles)</td>
</tr>
<tr>
<td>Phase III (deep anesthesia)</td>
<td>Regular, Abdominal. Minimal amplitude.</td>
<td>Eyeball position is fixed (in center). Reaction to light continues until mid-stage through this phase.</td>
<td>Coughing ceases. All muscles, including abdomen.</td>
</tr>
</tbody>
</table>

(Hall & Clarke, 1983)

(2) Injections
A. Location of injection
i. Subcutaneous injection (SC)

Subcutaneous injections are usually administered behind the base of the ear, but with piglets they are also administered in a hind leg.

ii. Intramuscular injection (IM)

Intramuscular injections are administered in either the neck or the rump, where there is a lot of muscle and little fat. Care must be taken to prevent the tip of the needle from entering the subcutaneous adipose layer. While aspirating air into the syringe, check to see that there has been no reverse flow of blood into the syringe.

iii. Intravenous injection (IV)

One of three veins [lateral auricular (ear vein), jugular (or cranial vena cava), or median caudal (tail vein)] is used for intravenous injection. If using the cranial vena cava, search for the depression located between the manubrium sterni and the point of the right shoulder. Place the tip of the needle in the center of the depression and insert it in the direction of the dorsal end of the opposite scapula. The tip of the needle will enter the large vein between or just in front of the first pair of ribs. The depth of the vein below the skin depends on the size of the pig (as a general rule, 25 kg → 4 cm; 26-50 kg → 6 cm; 51-80 kg → 8 cm; 81 kg or more → 10 cm).

iv. Epidural injection (EPI)

This method is also known as lumbar anesthesia. Find the depression between
the last lumbar vertebra and the first sacral vertebra, which is located around the intersection of the midline and the line that connects the left and right iliac bones. Place the needle tip immediately behind the spiral process of the last lumbar vertebra and insert it at a 20-degree angle toward the rear. Alternatively, place the needle tip in the center of the depression and insert it at a 45- to 60-degree angle toward the front.

3. Preanesthetic medication

Preanesthetic medication is used for several purposes, such as: to avoid excessive salivation, bradycardia, and other effects of an overly excited parasympathetic nervous system; to sedate the animal to facilitate administration of anesthetics; to enable the animal to recover gently from anesthesia; and to reduce anesthetic dosage. Particularly when anesthetics are administered by intravenous injection, it is best to administer preanesthetic medication intramuscularly.

i. When the following preanesthetic medications are not used in combination with other drugs, the dosages are as follows:

a. Atropine sulfate (0.025-0.05 mg/kg, SC or IM)
   Administered 15 min prior to administration of anesthetic. Must be used in combination with other drugs.

b. Azaperone (4% solution, 1.25-2.50 mg/kg, IM)

c. Ketamine hydrochloride (10-20 mg/kg, IM)
   Be careful not to inject ketamine hydrochloride into the adipose layer.

d. Sodium pentobarbital (IV)
   For animals weighing 30 kg or less, dosage is 29 mg/kg (30-50 kg → 24 mg/kg; 50-100 kg → 20 mg/kg; 100-300 kg → 10 mg/kg). Sodium pentobarbital dosage can be reduced by half when used in conjunction with acepromazine, for which the dosage is 0.03-0.1 mg/kg (IM). Sodium pentobarbital may cause respiratory suppression or arrest in animals weighing 100 kg or less.

e. Thiopental sodium (IV)
   For animals weighing 35 kg or less, dosage is 11-19 mg/kg (35-100 kg → 8-15 mg/kg; 100 kg or more → 1-7 mg/kg). A 2.3% intravenous injection is the usual method of administration. For large pigs this means a large injection; if thiopental sodium is injected too rapidly it could cause respiratory failure.

f. Sodium thiamylal
   6.6-11 mg/kg (IV)

ii. The following combinations of preanesthetic medications can be used to achieve excellent sedation.

a. Intramuscular injection of atropine sulfate (0.05 mg/kg) and azaperone or xylazine (2 mg/kg), followed 10-15 min later by ketamine sulfate (10-20 mg/kg).

b. Intramuscular injection of atropine sulfate (0.025 mg/kg) and xylazine (2 mg/kg), followed 15 min later by ketamine sulfate (5 mg/kg) and butorphanol (0.22 mg/kg).
XII. Anesthesia

[Administration of yohimbine (0.1 mg/kg) enables the animal to recover quickly from anesthesia.]

c. Atropine sulfate (0.025 mg/kg), medetomidine, (0.085 mg/kg), and butorphanol (0.22 mg/kg). Butorphanol enhances the sedative effect of medetomidine.

C. Intravenous anesthesia

Although inhalant anesthetics are safer than those administered intravenously, inhalants are often difficult to use in the field. It is necessary to develop safe and effective IV anesthetics. The following two methods of intravenous anesthetization are now being used for laparotomy operations.

a. Sodium thiamylal

Three or four ampules of sodium thiamylal (0.5 mg per ampule; 1 or 2 ampules will render a pig unconscious) are injected one at a time into the ear vein at intervals of 20 to 30 min. After each injection, leave the needle in the blood vessel for the next injection. To prevent the needle from causing blood to clot, fill it with heparin and use adhesive tape to hold it in place.

b. GGE

In 500 ml of 5 % glucose solution, dissolve GGE at 5% concentration. Add 500 mg of ketamine, 50 mg of xylazine, and mix. Administer by means of intravenous drip.

D. Epidural anesthesia

a. Lidocaine hydrochloride

Administer 1 ml of a 2.0% lidocaine hydrochloride solution for each 4.5 kg of body weight (max. 20 ml).

b. Procaine hydrochloride

Administer 2.0% procaine hydrochloride solution. For pigs weighing 45-50 kg, the dosage is 0.4 ml/kg. For pigs weighing 100 kg, it is 0.2 ml/kg. Maximum dosage is 20 ml.

(3) Inhalation anesthesia

Inhalant anesthetics are taken into the body through inhalation of an anesthetic gas. When the required intracerebral concentration is reached, the central nervous system is suppressed, the animal loses consciousness, muscles relax, and an analgesic effect is obtained. This method of anesthesia allows for regulation of the level of anesthesia throughout a long surgical procedure.

Relaxation of the masseter muscle and loss of reflexes in the larynx and pharynx due to induction of anesthesia can result in blocked airways, or can cause aspiration pneumonia. To solve these problems, it is common to insert a tube into the trachea (endotracheal intubation), but this is difficult to do with pigs, so a gas mask may be used instead.
A. Instruments, equipment, drugs, etc.

1. One (triple-gas) inhalation anesthesia machine (closed circulation)
2. Vaporizer for volatile anesthetics: Halothane and isoflurane can be combined. (If replaced frequently, the vaporizer should be rinsed with ether and dried.)
3. Soda lime: Needed for absorption of carbon dioxide. When color changes, soda lime must be replaced immediately.
4. Inhaler mask: The inhaler mask is made by cutting a large plastic bottle in half and using the upper section. The mouth of the bottle is attached to a rubber hose, and approximately six holes are pierced in the bottle near the edge where it was cut in half. Can also be used to anesthetize large dogs. (See Chapter V, "Surgical Procedures").
5. Tape to hold inhaler mask in place
6. Laughing gas: When laughing gas is used in combination with halothane, or when halothane is used alone, cardiac output and mean blood pressure are not reduced as greatly, and there are reports of increased blood flow to the internal organs.
8. Isoflurane: Does not cause the negative side effects brought on by halothane (depressed liver function, malignant hyperthermia). Has no observed side effects when used alone.

B. Administration of anesthesia

1. After starvation, deprivation of water, and sedation by means of preanesthetic medication, place the animal on the operating table.
2. After removing the tongue from the mouth, place the inhaler mask snugly over the snout so that the pig breathes through the mask.
3. Tape the tongue and mask to the skin so that the tongue does not return to the mouth and the mask does not slip out of place.
4. During induction of anesthesia, set laughing gas to 3%, oxygen to 1%, and halothane to 4%.
5. During maintenance of anesthesia, set laughing gas to 1-2%, oxygen to 1%, and halothane to 1-2%.
6. Isoflurane can be used in place of halothane. Isoflurane should be set to 2% during induction of anesthesia, and thereafter gradually increased to 4%.
7. Use of laughing gas is optional.
8. When laughing gas is used, oxygen concentration must be set to at least 20%.

(5) Acupuncture

Acupuncture has been used in China since ancient times to treat disease. More recently, electroacupuncture has been developed, in which an electric current is applied to the needles to increase their effect. Acupuncture has an analgesic effect, for which reason it is used as a form of anesthesia. It has no muscle relaxant effect. Electroacupuncture has been used in conjunction with epidural anesthesia for caesarian section operations (Ibaragi Prefectural Animal Experimental Farm).
(6) Responding to emergency

A. Respiratory failure

1. Stop administering anesthetics immediately.
2. Press repeatedly with force straight downward on the chest at a rate faster than that of natural breathing.
3. Administer oxygen at the rate of 5-8 liters/min for 4-5 min. Once the animal begins to take in air, reduce the volume of oxygen. Once normal breathing has resumed, reduce the oxygen supply to 1-2 liters/min.
4. If using barbital, in addition to the aforementioned steps, either administer doxapram hydrochloride (5-10 mg/kg) intravenously or give an alkaline transfusion.

B. Cardiac arrest

Cardiac arrest will result in death if myocardial activity is not restarted within 3 min.

1. If cardiac arrest is the result of suppression of myocardial activity, administer epinephrine (Bosmine) via intravenous injection (0.02-0.03 mg/kg).
2. If cardiac arrest is the result of fibrillation due to abnormal excitement (cardiac arrest during administration of inhalant anesthetics), administer 1 ml of 1% lidocaine hydrochloride via intravenous injection.
3. Regardless of the cause of cardiac failure, administer an alkaline transfusion.

C. Shock

The symptoms of shock include increased heart rate due to peripheral circulation failure, cyanosis, oliguria, hypothermia, accelerated respiratory rate, partial loss of consciousness, lethargy, and metabolic acidosis.

1. Thoroughly ventilate the animal, and administer oxygen.
2. In the case of anemic shock, rapidly administer a lactated Ringer's solution intravenously (20-40 mg/kg).
3. In the case of cardiac shock, administer isoproterenol via intravenous drip.
4. It is also effective to administer an α-adrenoreceptor antagonist by infusion, and corticosteroids (dexamethasone, 1-10 mg).
5. Shock is a defensive reaction to hypothermia. Take measures to maintain body heat, but do not make the animal too hot.

D. Malignant hyperthermia

The animal will die quickly if body temperature rises to 42-44°C. Dantrolene (Dantrium®) lowers body temperature effectively.

4. Other precautions

The autonomic nervous system in pigs is very sensitive, and pigs will faint when highly excited or subjected to sharp pain. Anesthesia is especially dangerous for obese pigs. Commercial pigs have a relatively small lung capacity and tend to become in oxygen deficit; thus, anesthesia is dangerous when the nose or larynx is obstructed, there are cardiac or pulmonary irregularities, or the pig has a vitamin or mineral deficiency. After anesthesia, if the pig is to be slaughtered for meat, bear in mind that there is a period of drug withdrawal.
Cited reference


Further suggested readings

Appendix 1. Non-invasive Embryo Transfer

Non-invasive swine embryo transfer techniques with a high degree of repeatability have recently been reported in both Japan and abroad, and there is growing hope that these methods will gain widespread application. According to these reports, non-invasive transfer does not yet match the pregnancy and survival rates of surgical techniques, but labor and costs are much lower than for laparotomy operations. Thus there is great interest in further improvement of non-invasive techniques. In this appendix we shall briefly describe the methods used by workers at the Tottori Swine and Poultry Experimental Station, who have been pioneers in this field in Japan. (This appendix is based on the "Manual for transcervical pig embryo transfer", by Isamu Yonemura, a researcher at the Tottori Swine and Poultry Experimental Station.)

<Required equipment and reagents>

<table>
<thead>
<tr>
<th>Product name</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiral catheter</td>
<td>For non-invasive transcervical embryo transfer.</td>
</tr>
<tr>
<td></td>
<td>Niwashiki (FHIK) for artificial insemination of pigs.</td>
</tr>
<tr>
<td></td>
<td>Fine-gauge catheter--for first pregnancy; Large-gauge catheter--for second and subsequent pregnancies. Beginning at the end of the spiral portion, use an oil-based marker to place marks at 5 cm intervals. Wash and dry the catheter, spray it with silicon spray, place it inside its sheath, and carry out gas sterilization.</td>
</tr>
<tr>
<td>Silicon spray</td>
<td>For coating the surface of the spiral catheter.</td>
</tr>
<tr>
<td>Catheter sheath</td>
<td>For sheathing the catheter during insertion. The sheath is too small</td>
</tr>
<tr>
<td></td>
<td>for the catheter used with second and subsequent pregnancies; for such pregnancies, use a plastic bag in place of the sheath.</td>
</tr>
<tr>
<td>3-way valve</td>
<td>For switching between injection of embryos and transfer fluid.</td>
</tr>
<tr>
<td>50 ml syringe</td>
<td>For injection of transfer fluid.</td>
</tr>
<tr>
<td>1 ml syringe</td>
<td>For injection of embryos.</td>
</tr>
<tr>
<td>5 ml tube (Falcon 2003)</td>
<td>For short-term preservation and transport of embryos. Place in temperature-controlled water bath at 37°C.</td>
</tr>
<tr>
<td>Polystyrene box</td>
<td>For transport of transfer fluid and embryos (from the laboratory to the transfer site).</td>
</tr>
<tr>
<td>Plastic bag</td>
<td>For transport of transfer fluid and embryos.</td>
</tr>
<tr>
<td>Thermal insulation material</td>
<td>For transport of transfer fluid and embryos. Warm the thermal insulation material and place it in a polystyrene box.</td>
</tr>
<tr>
<td>Transfer solution</td>
<td>Adding serum to PBS.</td>
</tr>
<tr>
<td>Stereoscope</td>
<td>For searching for embryos.</td>
</tr>
<tr>
<td>35 mm laboratory dish (Falcon 1008)</td>
<td>For searching for embryos.</td>
</tr>
<tr>
<td>PP catheter</td>
<td>Fitted to 1 ml syringe and used for picking up embryos (through suction).</td>
</tr>
<tr>
<td>18 G injection needle</td>
<td>With cap on, serves as cap for syringe.</td>
</tr>
<tr>
<td>Transfer fluid</td>
<td>Dulbecco's PBS + 10% FBS + 0.4 mg/ml amikacin sulfate(We have used in transfer medium)</td>
</tr>
<tr>
<td>Clean bench</td>
<td>For preparation of culture medium.</td>
</tr>
</tbody>
</table>
Appendix 1. Non-Invasive Embryo Transfer

☐ CO₂ incubator For culturing/preservation of embryos.
☐ Culture medium For culturing/preservation of embryos.
☐ Sterilization filter For sterilization of solutions using pressurized filter.
☐ 10 ml syringe For filter sterilization of various solutions.
☐ Embryo manipulation pipette For washing embryos. Made from a Pasteur pipette.
☐ Multiwell plate For culturing of embryos.
☐ Mineral oil To prevent evaporation of culture medium.
☐ 70% ethanol Kept in wash bottle, used to wash lab table, instruments, etc.
☐ Rope For holding the pig.
☐ Ampicillin Antibiotic for embryo recipient. 0.75 g per animal.
☐ PBS or Ringer’s solution For washing external genitalia and the catheter.
☐ Record book For keeping records.

1. Short-term preservation of embryos and preparation of the transfer syringe

For short-term preservation, Dulbecco’s PBS (-) + 20% FBS is kept in a 5 ml polystyrene tube (Falcon 2003) placed in warm water (37°C). The transfer embryos are placed in a plastic dish measuring 35 mm in diameter and gathered in the center of the dish. The embryos are picked up by suction using a 1 ml syringe fitted to a PP catheter. The fluid containing the embryos is sandwiched between air pockets, just as is done when a straw is used.

2. Preparation of transfer fluid

Approximately 30 ml of transfer fluid (at 38°C) and 10 ml of air are drawn into a 50 ml syringe to which an 18 G needle has been attached.

3. Transport of embryos

As shown in Appendix Figure 1-1 (page 109), place a new needle and cap on the 50 ml syringe, place the syringe in the plastic bag in which it was wrapped, then place the syringe and bag in a polystyrene box filled with thermal insulating material warmed to 39°C. The embryos can now be transported to the embryo transfer site.

4. Preparation of the embryo recipient

Begin starvation on the day of the embryo transfer (in the morning). Administer ampicillin intramuscularly (0.75 g per animal) on the day of the transfer and the day after. It is important that the pig be held securely in place. Using a non-slip rope made of cotton or hemp, restrain the snout and raise the pig’s head slightly higher than the dorsal line. The length of rope from the snout to the tying position is kept as short as possible. Standing on the right side of the pig, hold the tail and press the pig against the wall on the left to prevent it from falling or moving. During the embryo transfer, the pig often moves suddenly when the catheter begins to be inserted into the cervical canal. If the pig moves, the surgeon and assistant synchronize their movements to return the pig
Appendix 1. Non-invasive Embryo Transfer

to its original position. It is essential that the pig be standing in proper position when the embryos and transfer fluid are injected.

5. Transfer procedure

1. The assistant washes the external genitalia with lukewarm water, wipes them dry with paper towels, cleans them with an alcohol swab, pours sterilized PBS or Ringer's fluid over them, and wipes them again with a paper towel.

2. Thoroughly moisten the inside and outside of a spiral swine artificial insemination catheter (inside a sheath for bovine embryo transfers) with sterilized PBS or Ringer's solution. Place the catheter into the vagina without rotating it, and insert it to a point just short of the cervical canal.

3. Pull away the sheath to expose the tip of the catheter (Appendix 1 Photo 1).

4. While slowly rotating the catheter in a counterclockwise direction, continue inserting it into the uterus. At this stage of the estrous cycle (unlike the stage at which artificial insemination is carried out), any attempt to force the catheter further in than it can go will cause bleeding. Be careful to avoid doing this. Pregnancy has never been achieved after the occurrence of bleeding. The pig always moves. When this happens, return it to the correct position and continue to insert the catheter slowly.

5. Insert the catheter to the isthmus of the cervical canal and pull lightly on it to check the resistance. Once it has passed through the cervical canal, insert it into the uterine body.

6. Fill a three-way valve to the catheter, and the 50 ml syringe to one of the openings on the three-way valve. Slowly inject 10-15 ml and monitor for approximately 30 sec to be sure no fluid leaks back out. If any fluid leaks out, either re-insert the catheter or switch to a different embryo recipient (Appendix 1 Photo 2).

7. Fill the 1 ml syringe holding the embryos to the other opening on the three-way valve. Switch the flow paths and inject all of the fluid into the catheter. Then match flow paths of each syringe. Suck the fluid into the 1 ml syringe and inject it back into the catheter; repeat once (Appendix 1 Photo 3, Appendix Figure 1-2).

8. Deposit the embryos deep in the uterus by slowly injecting the remaining 10-15 ml of transfer fluid.

9. To flush out all liquid remaining the catheter, inject the 10 ml of air still remaining in the syringe.

10. Hold the catheter in place for 1 min, then remove it.

11. Untie the pig and return it to the holding pen. Move slowly to avoid startling the animal. Place an appropriate quantity of feed in the holding pen and allow the pig to stand still for a while.

12. Wash out the catheter, syringes, and three-way valve with transfer fluid and check whether any embryos were left in them.

Note: Room temperature must be maintained at 25°C or higher during embryo transfer. During cold weather, perform the procedure in a small room where the temperature can be controlled.
Appendix 1. Non-invasive Embryo Transfer

Appendix 1 Table 1. Pregnancy rates in transcervical embryo transfer are determined by a combination of two factors: a) whether the catheter has penetrated the isthmus of the cervical canal, and b) whether there is resistance when the transfer fluid is infused.

<table>
<thead>
<tr>
<th>Transfer conditions</th>
<th>No. of embryos transferred</th>
<th>No. of pregnancies</th>
<th>Pregnancy rate (%)</th>
<th>Avg. litter size</th>
<th>Implantation rate (%)</th>
<th>Avg. catheter depth (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canal penetrated/No resistance</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>3.0±1.55*</td>
<td>13.7±7.68</td>
<td>36.3±3.44b</td>
</tr>
<tr>
<td>Canal not penetrated/No resistance</td>
<td>10</td>
<td>6</td>
<td>60.0</td>
<td>3.0±0.89</td>
<td>20.9±6.74</td>
<td>27.9±3.45c</td>
</tr>
<tr>
<td>Canal not penetrated/Resistance</td>
<td>9</td>
<td>4</td>
<td>44.4</td>
<td>3.3±2.63</td>
<td>13.7±7.68</td>
<td>25.9±3.92d</td>
</tr>
</tbody>
</table>

Total 25 16 64.0 3.1±1.57 16.7±7.60 29.0±5.93

* Avg. value +/- standard deviation
a, b Statistically significant (P<0.01; Student's t-test)

Appendix 1 Photo 1 Once the spiral catheter is inserted to the cervical canal, slide off the plastic sheath and insert the tip of the catheter into the canal.

Appendix 1 Photo 2 Inject a small amount of PBS and check whether it leaks back out.

Appendix 1 Photo 3 The embryos in the 1 ml syringe are injected into the uterus, after which all the PBS is infused.
Appendix 1. Non-invasive Embryo Transfer

Appendix Figure 1-1 Transporting embryos and transfer fluid

Appendix Figure 1-2 Injecting embryos and transfer fluid
Further suggested readings

Appendix 2. Cryopreservation of Embryos

Pig embryo cryopreservation techniques have not yet been established, and the production of piglets from frozen embryos is generally low. Nevertheless, the stepwise method has produced three pregnant sows out of four transferred and the direct transfer method has yielded success with two pregnancies out of two sows transferred at the Central Research Institute for Feed and Livestock ZEN-NOH in Japan. In this appendix, we shall describe procedures followed at this Institute. Just as with other species, the stage of embryo development affects the success of cryopreservation of pig embryos, but it is generally agreed that survival has not been reported for embryos frozen prior to the blastocyst stage. For this reason, embryos used in cryopreservation experiments must have developed to the expanded blastocyst stage or beyond. However, considering the transmission of diseases and practical application, it will be necessary to develop cryopreservation techniques for expanded blastocysts rather than hatched blastocysts.

Because of the need to ensure disease-free embryo transfers, the Central Research Institute for Feed and Livestock ZEN-NOH does not use embryos which are hatching or have already hatched from the zona pellucida (including those which hatch during culturing), nor does it use embryos with a damaged zona pellucida. After collection, embryos are cultured for two to three hours before being frozen. The conditions of the culture medium are as follows: M199 + donor serum with a 5% iron supplement; 38.5°C; 5% CO₂; 5% O₂; 90% air.

<Required equipment and reagents>

<table>
<thead>
<tr>
<th>Product name</th>
<th>Purpose and method of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Programmable freezer</td>
<td>For cryopreservation of embryos. Ethanol bath type (Tokyo Rika Pro-cool, NBC-3400; equivalent to the Fujihira ET-1)</td>
</tr>
<tr>
<td>Liquid nitrogen</td>
<td>For preservation of frozen embryos.</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>Used during freezing of embryos. Also used as freezer lid.</td>
</tr>
<tr>
<td>Crystal mini-straw</td>
<td>Container for embryos during freezing.</td>
</tr>
<tr>
<td>Polysealer</td>
<td>For sealing the straw.</td>
</tr>
<tr>
<td>Semen straw (fine gauge, 0.5 ml)</td>
<td>For tagging the freezing straw.</td>
</tr>
<tr>
<td>Aluminum cane</td>
<td>Used as case for the straw.</td>
</tr>
<tr>
<td>PP test tube</td>
<td>Used as case for the straw.</td>
</tr>
<tr>
<td>Straw goblet</td>
<td>Used as case (lid) for the straw. Must have a hole cut in it to be used.</td>
</tr>
<tr>
<td>Liquid nitrogen tank</td>
<td>For preservation of frozen embryos.</td>
</tr>
<tr>
<td>Stereoscope</td>
<td>Used at maximum magnification (40-50x) for assessment of embryo quality.</td>
</tr>
<tr>
<td>Inverted microscope</td>
<td>Used for assessment of embryo quality.</td>
</tr>
<tr>
<td>Microscope photography equipment</td>
<td>For photographing the condition of embryos.</td>
</tr>
<tr>
<td>Warming plate for microscope</td>
<td>To keep embryos warm while under the microscope.</td>
</tr>
</tbody>
</table>
Appendix 2. Cryopreservation of Embryos

- Warming plate: To keep embryos warm while on the lab bench.
- Clean bench: For preparation of culture medium.
- CO₂ incubator: For culturing the embryos.
- Culture medium: For culturing the embryos. M199 + donor serum with a 5% iron supplement.
- Freezing solution: Medium for freezing of embryos. SW1: 1.5M glycerol + 1% dextran + 20% serum in M199. Direct: 4% ethylene glycol + 4% propylene glycol + 1% dextran + 20% serum in M199.
- Sterilization filter: For sterilization of culture medium through pressurized filtration.
- 10 ml syringe: For sterilization of culture medium through filtration.
- Embryo manipulation pipettes: For washing of embryos, etc. made from a Pasteur pipette.
- Multwell plate: For culturing of embryos.
- Mineral oil: To prevent evaporation of culture medium.
- 70% ethanol: Kept in wash bottle, used to wash lab table, instruments, etc.
- Record book: For keeping records.

1. Freezing procedures

1. Preparation of the freezing straw
   - Use a marker to draw marks on a crystal mini-straw as shown in Appendix Figure 2-1 (page 118). Use the body of a marker or some other such implement to flatten the 0.5 ml tag straw and write any necessary information on the top part of the straw.

2. Preparation of the freezing solution
   - Pass the freezing solution through a sterilization filter. Fill one well with fluid for washing and one well for fluid to be sealed in the freezing straw.

3. Sealing embryos in the freezing straw
   - After washing the embryos once (in the washing well), transfer them to the sealing well and seal them in the straw (equilibration time not required), with the embryos in the 2.5 cm portion of freezing solution in the center of the straw (see Appendix Figure 2-1, page 118). Seal the straw with a polysealer and attach the tag straw to it.

4. Placement in the programmable freezer; Ice seeding and freezing curves
   - Place a stand for small test tubes in the programmable freezer, then place a tagged freezing straw in the stand. Be sure the places where the straw has writing on it are not exposed to ethanol. Immediately after placing the freezing straw in the freezer, pull it out as far as the point where the upper part emerges from the ethanol. Using a pair of tweezers cooled in liquid nitrogen, grip the portion of the straw with freezing solution to carry out ice seeding. To keep the tag straw from dipping into the ethanol, cover the ethanol with a piece of polystyrene. The freezing curves for the stepwise method and the direct method are described on the following page.
Appendix 2. Cryopreservation of Embryos

**Stepwise method**

1. Place directly in freezer at -6°C and carry out ice seeding immediately (hold this temperature for 10 min).
2. From -6°C to -35°C at 0.6 °C/min (takes 48 min). Place in liquid nitrogen.

**Direct method**

1. Place directly in freezer at -6°C and carry out ice seeding immediately (hold this temperature for 15 min).
2. From -6°C to -32.5°C at 0.5 °C/min (takes 53 min). Place in liquid nitrogen.

(5) Placement in liquid nitrogen

Use a polystyrene box big enough to allow about 2/3 of the total length of the aluminum cane to be immersed in the liquid nitrogen. Place the aluminum cane in the liquid nitrogen and put the freezing straw in the polystyrene box. Place the straw in the PP test tube that is attached to the aluminum cane, and then cover the straw goblet with a lid. Be sure that the straw remains completely immersed in the liquid nitrogen when it is being moved. Because there is liquid nitrogen inside the PP test tube, there is little danger of the embryos being exposed to the air when they are being placed in the liquid nitrogen tank.

2. Thawing procedures

(1) Removing the straw

Remove the straw from the liquid nitrogen, keep it at room temperature for 5-10 sec, and then thaw the embryos in warm water (30-35°C).

(2) Stepwise method

Stepwise dilution is carried in the following five steps.

1. 1.0M glycerol + 20% FCS in M199 + 0.2M sucrose
2. 0.5M glycerol + 20% FCS in M199 + 0.2M sucrose
3. 0M glycerol + 20% FCS in M199 + 0.2M sucrose
4. 0.13M sucrose + 20% FCS in M199
5. 0.06M sucrose + 20% FCS in M199

After the embryos have been cultured for 2 h, those in which the blastocoele has recovered are deemed to have survived (and those in excellent condition are deemed transferable). These embryos are transferred.
(3) Direct transfer method

Carry out direct embryo transfer. When performing a surgical embryo transfer, suction the embryos into a PP catheter to carry out the transfer.

Further suggested readings

① Preparation of the straw

Use a Nozawa ZA176 crystal mini-straw (0.25 ml, washed and sterilized) with lines properly marked on it. Be careful not to touch the tip of the straw. Before using, wash once with freezing solution.

② Sealing of the straw

③ Tagging and sheathing straw before placement in liquid nitrogen

Flatten a 0.5 ml straw and write the date and ID number on the upper part. Fit it to a sealed straw and freeze.

To place the straw in liquid nitrogen, first put it inside an aluminum cane pre-cooled in liquid nitrogen, and then place the straw and an aluminum cane in the liquid nitrogen tank.
Appendix 3. Vitrification of Embryos

In comparison with the slow freezing method that has been used so far for
cryopreservation, vitrification has the advantage that it does not cause the formation of
ice crystals either inside or on the outside of embryos. On the other hand, time-
consuming procedures are required prior to freezing, and exposure to highly
concentrated cryoprotectants over a long period of time can damage embryos; thus, the
freezing procedure must be carried out with great skill. In Aichi prefecture, after vitrified
embryos are thawed, cryoprotectants are removed through a stepwise dilution process.
After these embryos were transferred surgically to recipients using a PP catheter,
pregnancy was achieved in two of three embryo recipients. In this appendix, we shall
describe this embryo transfer method.

1. Selection of embryos for freezing

The developmental stages of embryos suitable for cryopreservation range from
blastocysts just hatched to expanded blastocysts more than 200 μm in diameter.
Embryos collected on the sixth day after artificial insemination generally fall within this
range, and after freezing, thawing, and 48 h of culturing, more than 80% of them
survive. It is best to freeze embryos within 1-2 h after collection. Prior to freezing,
embryos are cultured temporarily in CZB medium containing 10% fetal calf serum (FCS)
(see Chapter X, Embryo Transfer, page 76) if kept in a 5% CO₂ incubator. If kept in an
incubator with only a warming function, embryos are cultured in HEPES-CZB (H-CZB)
medium containing 10% FCS.

2. Cryoprotectants and removal solutions

The basic solution of the cryoprotectant and its removal solution is PBS (m-PBS;
SIGMA). To prepare the cryoprotectant, take PBS, dissolve ethylene glycol (EG) in it,
then add 7% polyvinylpyrrolidone (PVP) at a concentration of 8 M (8 M EG in 7% PVP
solution). For the pre-treatment solution, use 2 M EG. For the removal solution, use 1.7
M galactose, and 1 M or 0.5 M EG.

3. Freezing procedures

(1) Pre-treatment of embryos
Place the embryos to be frozen in 2 M EG and keep them undisturbed for 5 min to
achieve equilibrium.

(2) Preparation of the freezing straw
Fill a 0.25 ml plastic straw with the fluid in the manner described below.
To draw the fluid into the straw, fit a 1 ml syringe to the end with the cotton plug.

(3) Sealing embryos inside the straw

Make 30-50 μl 2 M EG drops in a dish. After the embryos have been equilibrated for 5 min, transfer them to the drops. To pipette the embryos into the straw, first use the capillary effect to draw a small amount of cryoprotectant into a Pasteur pipette (fine tip, with mouthpiece attached). Then place the tip of the pipette in the microdrop, expel a small amount of cryoprotectant, and allow the capillary effect to pull the embryos into the pipette. Next, insert the tip of the pipette into the cryoprotectant inside the straw and transfer the embryos from the pipette into the cryoprotectant. Suction in 1.7 M galactose sandwiched between air pockets, and seal the end of the straw with sealer. The contents of the sealed straw are arranged as shown below.

(4) Placing embryos in liquid nitrogen

Holding the straw vertically with the sealed end down, place only the galactose portion in the liquid nitrogen. After the galactose freezes (takes 1-2 sec), place the straw in further to immerse the sections holding cryoprotectant, and continue slowly until the entire straw is immersed. If the entire straw is plunged quickly into the liquid nitrogen, it could crack. However, the entire process should be completed within 40 sec.
Appendix 3. Vitrification of Embryos

4. Thawing procedure, and removal of cryoprotectant

(1) Thawing procedure

Remove the straw from the liquid nitrogen, expose it to the air for 5 sec, then immerse it in water at 25°C for 10 sec.

(2) Removal of cryoprotectant

Make sure the solution inside the straw has thawed. Holding the straw by the sealed end, shake it vigorously to mix the contents. Next, cut off both ends of the straw and empty the cryoprotectant and embryos into a dish. Approximately 1 min after shaking the straw, transfer the embryos to 1 M EG and leave them undisturbed for 2 min. Wash twice with H-CZB, and then store them in H-CZB at 38°C until it is time to begin the transfer procedure.

5. Embryo transfer

In the case of surgical transfer, put the embryos into a PP catheter along with a small amount of H-CZB, and then carry out the transfer.

Further suggested readings

Appendix 4. Photographing Embryos

*Procedure for photographing with a Nikon automatic camera.*

1. Place the embryo to be photographed in a microdroplet of culture medium under paraffin oil (leave the dish uncovered).
2. Place the dish on the platform of the inverted microscope and position the embryo in the center of the field of vision (at low magnification).
3. Select the objective lens with the desired magnification power (100x, 200x).
4. When using color film, fit a blue filter to the light source. When using black-and-white film, fit a green filter to the light source.
5. Adjust the microscope so that the all four sets of double lines in the center are clearly focused. This will make it possible to take clear photos regardless of one's eyesight.
6. Adjust the magnification power, keeping the object to be photographed in the center of the square frame.
7. Adjust the focusing screen to the outer circumference of the embryo (or, if there is a zona pellucida, to the outer circumference of the zona pellucida).
8. Check light intensity.
9. If the embryo moves, wait until it is still.
10. The smaller the aperture, the deeper the point of focus, which is good for photographing an object with the thickness of an embryo.
11. Press the shutter button.
Questions and Answers

What are the most important points for a successful embryo transfer?

The following three points are very important.

1. Select a proper embryo recipient [Chapter IV].
   The estrous cycle of the sow chosen as the embryo recipient should lag 0-2 days behind that of the embryo donor. The embryo recipient should show strong signs of estrus, and its estrous cycles should finish quickly.

2. Transfer only morphologically sound embryos [Chapter IX].
   The embryos transferred should be morphologically sound, and should develop at the proper rate. Check to verify that sperm have attached to the zona pellucida.

3. Make sure the embryos are accurately placed in the reproductive tract of the recipient sow [Chapter X].
   Rather than worrying about preventing small amounts of air and culture medium from getting into the reproductive tract, it is more important to be sure that embryos are properly placed within the reproductive organs. For this purpose, use a hypodermic needle with the tip cut off or an embroidery needle to pierce a hole in an oviduct or the uterus, and check carefully to be sure that the tip of the needle is actually inside the reproductive tract.

Is it possible to collect porcine ovaries at the slaughterhouse and, as is done with cows, use the follicles and oocytes for in vitro maturation and fertilization, thereby producing pig embryos capable of normal development?

With current techniques, when follicles and oocytes are used for in vitro maturation and fertilization of pig embryos, the resulting embryos develop very poorly due to polyspermic fertilization.

What methods are used to test for pregnancy in the recipient sow after embryo transfer?

Some sows do not return to estrus even when the transfer does not result in pregnancy, so it is not sufficient merely to monitor for signs of estrus. There are several ways to detect pregnancy in its early stages. One method is to use ultrasonic doppler scanning to listen for a fetal pulse and heart functions. This method of detecting signs of
pregnancy is simple and accurate and, therefore, widely used (Product name: Preg-Doctor, Medata Systems LTD, UK; Importer for Japan: Nichiei Trading Corporation). In Canada, the device used is called Sonic-Preg®. Other methods include rectal examination, examination of the vaginal mucous membranes, etc. Testing early for pregnancy reduces costs by eliminating the need to keep an open sow for a long period of time after she fails to return to estrus.

I understand that the preanesthetic medication is administered via injection into the ear vein. Exactly how is that done?

Put the pig in a stall and restrain it as necessary. (Raise the ear vein by squeezing it between your thumb and index finger) If a winged (butterfly) needle set (21G3/4, Terumo, etc.) is used, a single person can administer the injection even if the pig moves around a little bit.

What sort of accidents can occur when using inhalation anesthetics containing halothane?

When an inhalation anesthetic containing halothane is used, a pig with porcine stress syndrome (PSS) may exhibit any number of irregularities (muscular twitching or rigidity; respiratory difficulty; irregular respiration; heart palpitations; raised body temperature; erythema interspersed with white splotches; etc.), and may even die. If these symptoms are observed, stop administering halothane immediately and terminate the operation. Some muscle relaxants (diphenylhydantoin) are effective in treating these symptoms. PSS is exhibited by pigs carrying the halothane-sensitive gene in their genome. Pigs in Japan have rarely shown signs of PSS in recent years, however, since the gene has apparently been bred out of most of the population.

When the purpose of embryo transfer is disease control, must the culture medium used for preservation and transport contain protein (blood serum or albumin)?

If embryos are placed in a preservation or transport culture medium that contains no macromolecules, the embryos tend to adhere to the walls of the container. If you do not wish to use a protein, polyvinyl alcohol (PVA) can be used as a substitute at a concentration of 0.1% to 1.0%.
We have administered hormones to pigs before, but it doesn't always result in normal estrus. Why is that?

There are different reasons, depending on whether the pig treated with hormones is a mature sow or a prepubertal gilt. In the case of prepubertal gilts, the animal selected must be as close as possible to puberty if good results are to be obtained. If the pig is too young, it will not respond well to the hormones. Even when hormones bring on estrus in a prepubertal gilt, the gilt does not always display the same standing heat that can be expected from a mature sow, although it is still possible to mate the gilt and obtain embryos. As for abnormal estrus in the case of mature sows, these animals will not respond to hormones if they are administered without regard to the natural estrous cycle. Routine monitoring of estrus is a fundamental requirement of embryo transfer work. For further information, see Chapter III "Preparation of the Embryo Donor".

There are other points to be considered as well. Prepubertal gilts, as mentioned previously, seldom display full-fledged standing heat, so it is necessary to monitor especially carefully for signs of estrus. It is also important to check the expiration date of the hormones administered, the preservation conditions, and the method of administration. Another key question is whether all of the prescribed dosage has been properly administered. It is sometimes necessary to check the nutrition of the embryo donor, and the manner in which it has been raised. There may be a problem with the pig; thus, all animals must be observed carefully on a routine basis.

Sometimes not even a single embryo is collected, even from a pig that has displayed normal estrus. Why is that?

One possibility is unsuccessful mating. Or, in the case of artificial insemination, it could be due to poor quality semen or inadequate skill on the part of the person performing the insemination. It is also necessary to ask whether the pig was inseminated at the correct time. The normal development of the embryos could have been affected by contamination entering the uterus during mating. Weather conditions at the time of mating also constitute an important factor. For example, it is thought that insemination failure and early embryonic death are more likely to occur in the summer. It may be necessary to check the nutrition of the embryo donor, and the manner in which it has been raised. Needless to say, the possibility of a mistake during embryo collection must be considered. In this regard, there is a particularly high risk of mistaking the timing of the end of estrus, which can result in embryos being collected before they have reached the required stage of development. When this happens, the wrong part of the reproductive tract may be flushed.
After the flushing fluid is recovered, it is sometimes cloudy or contains blood. Why is that?

When the recovered flushing fluid is cloudy, it could mean that the embryo donor has contracted a mild case of endometritis [The inability to collect embryos is considered a key symptom in the diagnosis of endometritis]. When embryos are collected from a slaughtered pig, the recovered flushing fluid often becomes cloudy if too much time elapses between slaughter and the start of flushing, but normal embryos can still be collected from such cloudy fluid. On the other hand, the most common cause of blood in the flushing fluid is damage to a blood vessel, having been caused by ophthalmological scissors or some other instrument when the hole was made in the uterine wall for insertion of the balloon catheter. This in itself does not harm the embryos, but blood in the flushing fluid does make the embryos harder to find. This prolongs the procedure, which may in turn lower the survival rate. If such cases, filtration of the fluid is recommended. Also, care must be taken during flushing not to cause bleeding in the first place.

It is difficult to determine the survival of collected embryos.

Currently, morphological examination is the simplest method for determining survival. As mentioned previously in the literature review, there appears to be a high positive correlation between morphology and survival after collection. If the photos of high-quality embryos provided elsewhere in this manual are committed to memory, they should be helpful for judging embryo quality. This method has shortcomings, however, since the judgment is very subjective and it takes a long time for the practitioner to gain proficiency in assessing embryo quality. There is a need to develop methods that offer more objective standards for assessing the survival and quality of embryos.

It is extremely difficult to remove the foreign matter attached to collected embryos.

The method of removal depends on what exactly the foreign matter is. Foreign matter attached to embryos immediately after collection is almost always bits of detached endometrium. This matter can be eliminated a little at a time by pipetting the embryos repeatedly with a Pasteur pipette, the tip of which has been modified to an inner diameter slightly larger than the embryos. This method yields relatively good results with embryos still inside the zona pellucida, but complete removal of foreign matter from embryos that have already hatched from the zona pellucida is difficult to achieve.
The textbooks indicate that embryos should reach specific stages of development at specific lengths of time after mating, but we do not always find this to be true. Why is that?

Estrus lasts longer in pigs than cattle, and ovulation occurs in the latter part of the estrous cycle. More so than with cattle, accurate determination of both the beginning and the end of estrus is important with pigs (see pp 18-19).

What is the difference in the degree of distillation required for distilled water used to prepare culture media as compared to distilled water used for final rinsing of surgical and laboratory instruments and equipment?

Distilled water used for final rinsing of surgical and laboratory supplies (especially those, such as containers for culture medium, which may come in direct contact with embryos) should basically be of the same quality as that used to prepare culture medium. Specifically, it should be super-purified water (prepared by passing deionized water through a millipore filter to achieve a relative resistance of ca. 18 ohms). Alternatively, it is possible to use deionized water that has been distilled at least twice in glass containers.

Stages of development sometimes vary, even among embryos collected from a single embryo donor. Can they all be considered normal as long as they are morphologically sound?

Such discrepancies do occur. When counting the last day of standing heat as day 1, for example, embryos collected from a single donor on day 6 sometimes vary from the 4-cell stage to the expanded blastocyst stage. Embryos collected at an early stage of development often fail to develop any further when cultured in vitro even when no morphological abnormality is observed (using an inverted microscope at regular magnification). When the correlation between developmental stage and number of days since standing heat differs greatly from that shown in Chapter II, Table II-5 (page 17), the embryo is probably abnormal.

Collecting every single fertilized eggs (or embryo) in the reproductive tract is important. When monitoring for estrus and carrying out artificial insemination, are there any points to be especially careful about?

Ovulation in pigs takes place during the latter half of estrus, often at the very end. To proper insemination, the end of standing heat should be confirmed. Fertilization rates
are best when artificial insemination is carried out during standing heat. Furthermore, knowing when standing heat began is particularly necessary for efficient pronuclear embryo collection. Knowing when standing heat began and ended is also necessary in order to collect embryos at the desired stage of development.

Do you have to be a veterinarian to perform pig embryo transfer?

Unlike the transfer of bovine embryos, pig embryo transfer techniques are not yet established in general practice. Pig embryo transfer is only carried out experimentally, and there is not yet any system for accreditation of embryo transfer specialists, as there is in the case of cattle. Because it is still at an experimental stage, it naturally follows that the mere fact that surgery is required does not mean that one must be a qualified veterinarian (especially for embryo collection), although we believe it is certainly best to be knowledgeable in the field of veterinary medicine. In addition, being a qualified veterinarian does not necessarily mean one is qualified to carry out pig embryo transfer. For anyone who intends to practice pig embryo transfer in the future, the most direct path to this goal would probably be to acquire the required skills and background knowledge in reproductive physiology at an institution with experience and a proven track record in the field. However, there are aspects of pig embryo transfer which must be performed by a qualified veterinarian (purchasing of antibiotics and anesthetics, for example); thus, pig embryo transfer in the future will undoubtedly be carried out at institutions which employ veterinarians.

Note: While the above is true for Japan, its status may differ in different countries.

When there are few pigs available for embryo transfer, estrous cycles have to be synchronized. We have heard that effective new drugs are being developed for this purpose. Could you describe them?

A pharmaceutical company called Intervet has developed a hormone called PG-600 [SDS Biotech is currently collecting clinical data required for the approval process for sale of this hormone]. In Japan, this product is called CG-600 to avoid confusion with a prostaglandin product already on the market. CG-600 is a mixture of refined eCG 400 IU and hCG 200 IU. When administered by injection to a prepubertal gilt, it can induce estrus very effectively, and subsequent occurrence of irregular estrus is reportedly very infrequent. However, it does little to stimulate superovulation. In mature sows, if CG-600 is injected when the concentration of progesterone in the blood begins to drop (as on the 12th day of the estrous cycle), estrus is reportedly induced a very high percentage of the time.
Uncollected embryos sometimes cause pregnancy in the donor sow. How can that be prevented?

A large number of embryos might go uncollected for a number of reasons. Improper flushing is one possibility. As well, if collection coincides with the migration of the embryos from the oviducts to the uterus, and only the uterus is flushed, embryos left in the oviducts could produce a pregnancy. Some of the oocytes may not be fertilized. In the former two cases, many donor sows will be at risk of pregnancy. Pregnancy can be prevented by injecting an iodine solution into the uterus after the flushing procedure is finished. The iodine solution has the effect of stimulating and renewing the endometrial cells and, thus, tends to hasten the onset of estrus. It may also enhance subsequent reproductive success. Take care, however, to prevent the iodine from leaking into the abdominal cavity, as this will cause adhesions of the internal organs.

Embryos collected on the 6th day or later sometimes turn out to be hatched blastocysts in a shrunken state. Why do they shrink? Is it all right to use these blastocyst in experiments?

This shrinkage is probably due to the shock of transfer from the uterus to in vitro environment, but the actual cause is not known for certain. Specifically, the shrinkage is thought to be caused by changes in the embryo’s microenvironment (temperature, osmotic pressure, pH, etc.). Something happens which has a direct effect upon the dynamics of the fluid inside the blastocoele. Very often, when such blastocysts are placed in culture media and cultured for a short time, the blastocoelites refill with fluid and the blastocysts regain their original form. Blastocysts which regain their original form can be treated the same as those which did not undergo shrinkage.

In the expanded blastocyst stage, is there a proportional relationship between expansion of the embryo’s diameter and increase in the number of cells?

Although we cannot base our conclusion on a large statistical base, there does appear to be a close relationship between the two. This does not mean, however, that embryos of the same diameter will necessarily have the same number of cells. This is especially true of embryos from different pigs. Furthermore, when this relationship is expressed in the form of a graph, the longer the embryos develop in culture, the more gentle will be the slope of the line representing proportionality between expansion of diameter and increased numbers of cells. Culturing in vitro may increase embryo size, but it has little effect on the rate of cell division.

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### Pig Embryo Collection

<table>
<thead>
<tr>
<th>Collection date</th>
<th>Day No. of estrus</th>
<th>Breed</th>
<th>Donor No.</th>
<th>Party</th>
<th>No. of operations</th>
<th>Weight</th>
<th>Birth (DD/MM/YY)</th>
</tr>
</thead>
</table>

**Other (Estrus, Clinical history, etc.)**

Superovulation (DD/MM, time, hormone, worker's name)

Estrus (DD/MM, time, <X = none; Δ = weak; O = reddened, enlarged; Z = standing heat>, monitor's name)

<table>
<thead>
<tr>
<th>Mating (DD/MM)</th>
<th>Time</th>
<th>Name of boar (breed)</th>
<th>amount of ejaculate</th>
<th>Sperm mobility</th>
<th>Natural/AI</th>
<th>Other</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Left</th>
<th>Right</th>
<th>Total</th>
</tr>
</thead>
</table>

- No. of corpora lutea
- Condition of ovaries, uterus
- No. of embryos collected

Stage Rank

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
</table>

Surgery notes (time, type of anesthetic, physical state, etc.)

<table>
<thead>
<tr>
<th>Induction of anesthesia commenced (____ : ____ o'clock)</th>
<th>Surgeon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance of anesthesia commenced (____ : ____ o'clock)</td>
<td>Assistant</td>
</tr>
<tr>
<td>Surgery commenced (____ : ____ o'clock)</td>
<td>Anesthesiologist</td>
</tr>
<tr>
<td>Embryo collection commenced (____ : ____ o'clock)</td>
<td>Embryo handling</td>
</tr>
<tr>
<td>Suturing commenced (____ : ____ o'clock)</td>
<td></td>
</tr>
<tr>
<td>Surgery completed (____ : ____ o'clock)</td>
<td></td>
</tr>
<tr>
<td>Embryo search completed (____ : ____ o'clock)</td>
<td></td>
</tr>
</tbody>
</table>

Slaughter notes (time of slaughter/embryo collection/completion of embryo search, etc.)
### Pig Embryo Transfer

<table>
<thead>
<tr>
<th>Transfer date</th>
<th>Day No. of estrus</th>
<th>Breed</th>
<th>Recipient No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parity</th>
<th>No. of operations</th>
<th>Weight</th>
<th>Birth (DD/MM/YY):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Other (Estrus, Clinical history, etc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hormone treatment (DD/MM, time, hormone, worker's name)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Estrus (DD/MM, time, &lt;X = none, △ = weak, ○ = reddened, enlarged; @ = standing heat, monitor's name)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Left</th>
<th>Right</th>
<th>Totals</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of corpus lutea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition of ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition of uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of embryos transferred</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day No. of estrus/classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Embryo donor/breed</th>
</tr>
</thead>
<tbody>
<tr>
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</table>

<table>
<thead>
<tr>
<th>Surgery notes (time, type of anesthesia, physical state, etc.)</th>
</tr>
</thead>
<tbody>
<tr>
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<table>
<thead>
<tr>
<th>Room temperature</th>
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<tbody>
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<table>
<thead>
<tr>
<th>Induction of anesthesia commenced (<em><strong>:</strong></em> o'clock)</th>
<th>Surgeon</th>
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<tbody>
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<table>
<thead>
<tr>
<th>Maintenance of anesthesia commenced (<em><strong>:</strong></em> o'clock)</th>
<th>Assistant</th>
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</table>

<table>
<thead>
<tr>
<th>Surgery commenced (<em><strong>:</strong></em> o'clock)</th>
<th>Anesthesiologist</th>
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<tr>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Embryo transfer commenced (<em><strong>:</strong></em> o'clock)</th>
<th>Embryo handling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<table>
<thead>
<tr>
<th>Suturing commenced (<em><strong>:</strong></em> o'clock)</th>
</tr>
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<tbody>
<tr>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Surgery completed (<em><strong>:</strong></em> o'clock)</th>
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<tbody>
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<table>
<thead>
<tr>
<th>Pregnancy test (results)</th>
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</thead>
<tbody>
<tr>
<td></td>
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<table>
<thead>
<tr>
<th>Farrowing date (results)</th>
</tr>
</thead>
<tbody>
<tr>
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</table>
**Transcervical Pig Embryo Transfer (Tottori Swine and Poultry Experimental Station)**

**Date (DD/MM/YY):**

<table>
<thead>
<tr>
<th>Embryo donor</th>
<th>Breed</th>
<th>ID No</th>
<th>Embryo age (days)</th>
<th>Embryo origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Purpose of experiment:**

<table>
<thead>
<tr>
<th>Breed</th>
<th>ID No</th>
<th>Birth date (DD/MM/YY)</th>
<th>Day No. of estrus</th>
<th>Weight</th>
<th>Parity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

**No. of days after standing heat:**

<table>
<thead>
<tr>
<th>Day</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
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</table>

**Estrus induction method:**

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>GtG; /</th>
<th>hCG; /</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ax, lot</td>
<td>ax, lot</td>
</tr>
</tbody>
</table>

**Date (DD/MM):**

<p>| | | | | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
</table>

**Estrus:**

**Mating notes:**

**Restraining method:**

**Method of anesthesia:**

**Transfer fluid:**

**Volume of fluid:**

**ml**

**Antibiotics:**

**Amount of antibiotics:**

**Transfer instruments/Catheter:**

**Other transfer instruments:**

**Time of transfer:**

**Room temperature:**

**Hemorrhaging:**

**Fluid leakage:**

**Fluid resistance:**

**Catheter rotation:**

**Catheter lock:**

**Catheter depth upon insertion:**

**Catheter depth upon removal:**

**cm**

**cm**

**Name of person who performed:**

Assistant: Assistant: Assistant:

**Embryo development:**

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Embryos transferred:**

**Total:**

**Embryos lost:**

**Return of estrus:**

<table>
<thead>
<tr>
<th>Day</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Pregnancy test performed by:**

**Pregnancy:**

**Parturition date (DD/MM):**

<table>
<thead>
<tr>
<th>/</th>
<th>/</th>
<th>Other</th>
</tr>
</thead>
</table>

**Size of litter:**

**Live births:**

**Still births:**

**Mummified fetuses:**

**Total:**

---

**Reference Material**
## Authors of this Manual

(in alphabetical order)

<table>
<thead>
<tr>
<th>Name</th>
<th>Address of Employment</th>
<th>Chapters contributed to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kameyama, Kenji</td>
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</tr>
<tr>
<td>Kashiwazaki, Naomi</td>
<td>Y.S. New Technology Inc. 519 Hanabayashi, Shimoishibashi, Ishibashi-cho, Shimo Toga-gun, Tochigi Prefecture, Japan</td>
<td>III, IV, VI, XII, Q&amp;A</td>
</tr>
<tr>
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</tr>
<tr>
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<td>V, XII, Note from the Editors</td>
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</tbody>
</table>

The editors wish to express sincere gratitude for the valuable information and ideas received from the following persons.

<table>
<thead>
<tr>
<th>Name</th>
<th>Address of Employment</th>
<th>Chapters contributed to</th>
</tr>
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<tbody>
<tr>
<td>Downey, Bruce</td>
<td>Department of Animal Science, McGill University, Macdonald Campus 21, 111 Lakeshore Rd, Ste Anne de Bellevue, QC, Canada H9X3V9</td>
<td>Supervising English translation</td>
</tr>
<tr>
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<td>English translation</td>
</tr>
<tr>
<td>Ito, Shougo</td>
<td>Nagano Prefectural Animal Experimental Farm 10931-1 Kataoka, Shioji City, Nagano Prefecture, Japan</td>
<td>Operating table photos</td>
</tr>
<tr>
<td>Kobayashi, Shoji</td>
<td>Aichi Prefectural Agricultural Research Center 1-1 Migasumi, Iwazukuri, Nagakute-cho, Aichi-gun, Aichi Prefecture, Japan</td>
<td>Appendix 3</td>
</tr>
<tr>
<td>Nakazawa, Yoshinori</td>
<td>Kanagawa Prefectural Institute of Animal Industry 3750 Hongo, Ebina City, Kanagawa Prefecture, Japan</td>
<td>Q&amp;A</td>
</tr>
<tr>
<td>Onishi, Akira</td>
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<td>Photos of surgical procedures, etc.</td>
</tr>
<tr>
<td>Ozawa, Eiko</td>
<td>Department of Animal Health, Bureau of Livestock Industry, Ministry of Agriculture, Forestry and Fisheries 1-2-1 Kasumigaseki, Chiyoda-ku, Tokyo, Japan</td>
<td>Q&amp;A</td>
</tr>
<tr>
<td>Yonemura, Isao</td>
<td>Tottori Swine and Poultry Experimental Station 106 Kinuya, Saimakssc-cho, Saimaku-gun, Tottori Prefecture, Japan</td>
<td>Appendix 1, Photos of non-invasive transfer techniques</td>
</tr>
</tbody>
</table>

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Closing Comments

This manual has not dealt with various embryo transfer techniques that are described in manuals for bovine embryo transfer, such as embryo splitting, in vitro insemination or fertilization, sexing, and nuclear transfer. The development of these techniques is clearly required for further advancement of the field of pig embryo transfer, but these cutting-edge techniques require the foundation of more basic techniques, e.g., embryo collection, transfer, and storage or preservation. In view of the tremendous technical challenges posed by pig embryo transfer, the primary purpose of this manual has necessarily been to focus on these latter, more basic techniques. We do hope, however, to deal with the more advanced techniques in future revised versions of this manual.

As revised versions are released, we also intend to add more content and make the manual easier to read and use. Any comments from readers would be most welcome, and can be directed to Toshiyuki Kojima, the National Livestock Breeding Center.

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Japanese Society for Development of Pig New Technologies
Manual on Pig Embryo Transfer Procedures, Editorial Committee