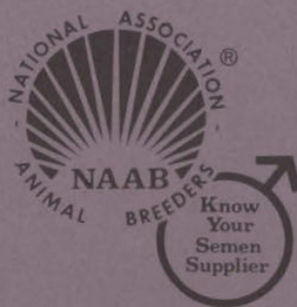


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HISTORICAL PERSPECTIVE OF AI: COMMERCIAL METHODS OF PRODUCING SEX SPECIFIC SEMEN, IVF PROCEDURES

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POLGE

Dr. Chris Polge received the BSc degree in Agriculture from the University of Reading and the Ph.D. degree from the University of London. His discovery in 1949 of a method for the preservation of sperm and other living cells at very low temperatures led to widespread developments. The greatest practical application has been in the deep freezing of semen for use in AI which revolutionized cattle breeding worldwide. For more than 30 years Chris Polge worked at the Animal Research Station in Cambridge, England, which was at the forefront of many developments in animal reproductive physiology. His own research, in addition to semen freezing, involved the development of AI in pigs, the control of reproductive cycles, fertilization and early embryonic development, in vitro fertilization and embryo transfer as well as embryo freezing and genetic manipulation. Dr. Polge has received numerous awards and honors. After retirement from government research service, he has been involved in establishing commercial opportunities for the application of research developments in animal breeding. He is now a scientific consultant to several companies.

Introduction

I should like to give my special thanks to the organizers of the Sixteenth NAAB Technical Conference for inviting me to talk at this meeting. I am very happy to be here because the last time that I gave a paper to the NAAB was in 1968 during the Second Technical Conference which was held then in Chicago. At that time it was easy for me to attend because I was spending a year in the

USA while on sabbatical leave from my laboratory at the Animal Research Station in Cambridge, England. During that year I worked for a few months with a very old friend and colleague, Dr. M.C. Chang, at the Worcester Foundation for Experimental Biology in Shrewsbury, Massachusetts. If one thinks of historical perspectives in research on animal reproduction, Chang is certainly a name that should be held in high esteem because of his many important contributions especially to the fundamen-

tal aspects of fertilization and early embryonic development. These laid the foundations of some of the modern technologies in animal breeding that are being developed today. I am therefore very glad to have had several opportunities to work with him in the past.

Most of my sabbatical leave, however, was spent at the University of Illinois in Champaign-Urbana with another good friend and colleague, Dr. Phil Dziuk, and with him too I have had a lot of good collaboration over the years. It was once said of AI that it was "a research that knew no frontiers" because its growth was much dependant on the free international exchange of ideas and information. I believe this to be true for many areas of research and sabbatical leaves are a great stimulus in this respect. It has been my experience that visits to other laboratories and having visitors to one's own laboratory are most rewarding. Certainly while living in Illinois for some time in 1968, opportunities were provided to visit a number of other universities and I got to know many of the people who were then, or have since become, leaders in the field of animal reproduction and artificial breeding in the USA. It is because of these friendships and the strong feeling of attachment for this part of America, that I am pleased to be back here today.

Historical Perspectives

My comments on historical perspectives of AI will relate mainly to some of my personal experiences. In my paper at the Second Technical Conference I described our work during the early 1950's on the discovery of a method for deep freezing of semen and its application in AI thereafter (11). It is salutary to think that a large proportion of the present audience were probably not yet born when we were carrying out those early experiments on semen freezing (13,14). The majority of people now take it for granted that sperm as well as many other types of living cells, can be frozen and stored successfully at very low temperatures. Yet cryopreservation of sperm in conjunction with AI has been hailed as a breakthrough without equal or in any other area of modern animal breeding. So historically, it certainly was a landmark.

The initial experiments on semen freezing and early application in AI were carried out in England, but a major contribution to more widespread application and to the safe and effective use of frozen semen was made in the USA. This was due primarily to the work of American Breeders Service (ABS) and the enthusiasm of its owner, J. Rockefeller Prentice. As soon as we had published the results of the first successful experiments on freezing bull semen, 'Rock' Prentice wanted it for ABS. He immediately grasped the significance of the huge advantages that could be gained with frozen semen in an organization such as ABS which then had several bull studs and a

widespread distribution network. 'Rock' Prentice invited me to come and work with ABS in Madison during the Fall of 1953 to assist in the development of their work. This was an interesting time for me and I remember that Hank Dunn was in charge of the semen freezing laboratory and Harold Hafs was with us as a technician. Thus, other early friendships were established and meetings were arranged between many of those who were involved in the development of AI in the USA.

In England we had been storing semen in solid CO₂ and alcohol at -79°C, although we had already shown that the sperm could survive exposure to the much lower temperature of liquid air. In the USA, however, liquid nitrogen was becoming much more readily available and the very significant contribution made by 'Rock' Prentice was to encourage the Linde Company in Chicago to develop effective containers for the storage and distribution of frozen semen in liquid nitrogen. Linde developed a 'super insulation' material which, when introduced into the vacuum space of stainless steel vessels, enabled them to manufacture very efficient containers for long term storage of liquid nitrogen. These soon became widely used throughout the industry and did much to aid the rapid growth in the use of frozen semen which then took place.

The impact of AI and frozen semen on dairy cattle improvement throughout the world has been achieved through the dissemination of semen from bulls of superior genetic merit. Historically, therefore, the establishment of effective genetic evaluation programs has been of the greatest importance. Progeny testing programs using large numbers of half sibs on numerous farms, have been greatly assisted by the use of AI and frozen semen. Multiple ovulation and embryo transfer (MOET) in selected nucleus herds has more recently been introduced to accelerate the rate of genetic gain and the efficiency of genetic selection may be improved still further in the future by the identification and use of genetic markers associated with quantitative traits.

Throughout the history of animal breeding, however, there has long been an elusive goal - namely that of being able to predetermine the sex of offspring that are born. This would have a huge influence on the efficiency and economics of animal production.

Shortly after the Second NAAB Technical Conference in 1968, I took part in another meeting that was organized by the University of Wisconsin College of Agriculture and Life Sciences in collaboration with American Breeders Service, entitled 'Artificial Breeding in the Age of AI'. In my paper at that meeting (12) I identified the sexing of semen as one of the next most important steps in artificial breeding, but the prospects did not seem good. Recent work at that time on the use of a sedimentation procedure for the attempted separation of sperm with an X or Y chromosome had proved to be

ineffective. In fact, over the years there had been many reports and claims to the development of a variety of physical procedures for the separation of sperm to achieve an alteration in sex ratio, but none proved to be repeatable. When in Illinois in 1968, I remember discussing semen sexing with Dr. Andy Nalbandov and the frustration of the apparent ineffectiveness of attempted separation techniques. He described semen sexing as the 'crock of gold at the end of the rainbow' - in other words, he saw it as a prize of great value which might always remain beyond our grasp. In the ensuing years, however, this prize has indeed become obtainable and the ability to preselect the sex of offspring in farm animals through the use of separated X and Y chromosome bearing sperm based on differences in DNA content is now a reality (6).

In contrast to the many previous attempts to separate sperm for sex determination on the basis of potential or perceived physical differences, DNA content is the only scientifically validated and measurable difference between sperm with an X or Y chromosome. The development of a reliable technology for semen sexing has been dependant on advances in DNA analysis of sperm and developments in flow cytometry/cell sorting techniques and instrumentation (5). One of the most crucial steps was the modification of a laser-based flow cytometer for high resolution DNA analysis of sperm (8). Because of the small differences in DNA content between X and Y sperm in farm animals (3-4 percent) and the fact that the sperm heads are paddle shaped, differences in intensity of fluorescence of sperm stained with fluorochromes can only be detected accurately when the laser beam passes through the flat surface of the sperm. Modification of the flow cytometer to determine and detect orientation of the sperm to the laser beam was therefore an important advance. Further work on improved methods of staining and on the protocols for sperm treatment led to the ability to sort semen into relatively pure populations of X and Y sperm that retained viability. Convincing evidence that sex preselection had come of age was the birth of young from rabbits following insemination with semen enriched for X or Y sperm (6). The sex ratio of the offspring produced reflected almost precisely the ratio of X and Y sperm in the semen used for insemination. I remember writing to Larry Johnson after the publication of this paper to congratulate him and to say 'this is it'. The crock of gold had been grasped. But it seemed likely that there was still much to be done before the technology could be used effectively in farm animal breeding, particularly for cattle.

Steps Towards Commercial Application/IVF

One of the most serious limitations to the use of sexed semen in animal breeding is the small number of sperm available for insemination due to the slow rate at which it

can be sorted. This is the result of the flow rate that can be used for viable sperm, orientation of the sperm to the laser beam and the restricted setting of sorting windows necessary to achieve relatively pure samples of viable X and Y sperm. In practice it has been found that to achieve a purity of around 90 percent for X and Y sperm, $3-5 \times 10^5$ sperm can be sorted per hour (2). This is obviously not enough for any extensive use in routine AI. The production of offspring in rabbits was achieved by surgical insemination of small numbers of sorted sperm into the oviducts (6) and surgical insemination with sexed semen has also been used in pigs (4). In cattle, however, *in vitro* fertilization (IVF) seemed the most appropriate route for application, especially since embryo culture had been well developed in this species (9).

Our company had a long experience in the production of cattle embryos by *in vitro* techniques and also in commercial application. A collaborative research and development program was therefore established with the USDA to develop the Beltsville semen sexing technology in cattle breeding. For me, personally, this was also a happy collaboration because it provided an opportunity to renew work with Dr. Larry Johnson who was a friend of long-standing. Our friendship dated back to the time when we had a mutual interest in developments in pig AI and frozen boar semen.

The program has now been running for several years and during this time considerable progress has been made towards practical applications. Experience has been gained in the production of cattle embryos of predetermined sex by *in vitro* techniques and the use of these embryos on commercial farms resulting in the birth of calves (2,3).

Sperm Sorting

In the first instance it was shown that the sorting technology developed at Beltsville could be repeated successfully at another location and with different equipment. A flow cytometer/cell sorter (Becton Dickinson, FACStar) was set up at our IVF laboratory in Cambridge, England and modified for sperm sorting by provision of a beveled injection needle and second fluorescence detector located at 0° to the laser. Semen was incubated at 35°C with the vital fluorochrome, Hoechst 33342, in order to obtain uniform staining and sorted to give samples of X and Y sperm of 80-90 percent purity. The sperm were collected into a suitable buffer to maintain viability (6). In practice, a period of two hours sorting provided approximately 8×10^5 X and Y sperm for IVF. The purity of the samples was validated by reanalysis of aliquots of the sorted sperm in the cell sorter (7).

IVF and Embryo Production

After sorting, the sperm were washed and concentrated by centrifugation in preparation for IVF. Oocytes were obtained from slaughterhouse ovaries and standard techniques used for maturation, IVF and embryo culture (9). Approximately 2000 sperm per egg were used in the IVF droplets. The sorting procedure did not markedly affect motility and acrosomal integrity of the sperm and a fertilization rate of around 80 percent was obtained for both X and Y sperm. There was, however, a marked effect of the dye on the rate of embryonic development. Embryos produced from sorted sperm developed more slowly than controls and blastocyst production following culture was reduced to 17 percent as compared to 35 percent for controls (1). The sex of embryos produced, however, as determined by DNA probe and PCR, reflected closely the ratio of X and Y sperm in the samples used for IVF (3).

Embryo Transfer

In a preliminary study at Cambridge, 18 embryos produced following IVF with semen sorted for X and Y sperm were transferred as twin embryos to nine recipients. Four cows calved, producing two sets of twins and two single calves, all of which were of the predicted sex (2).

A larger scale field trial was then carried out. Semen sorted for Y sperm to a purity of around 90 percent was used for IVF and Grade One blastocysts produced after 7-8 days of culture were frozen in media containing ethylene glycol so that they could be used later for direct transfer to recipients. Twin transfers to 96 recipients were conducted on 11 commercial farms in Scotland and this resulted in 41 calves from 35 calvings; 37 were males and four were females. Thus, the sex ratio was as predicted and all the calves were normal (3).

Future Developments

It is clear that semen sexing involving the separation of X and Y chromosome bearing sperm based on DNA content is an accurate and repeatable technology, and offspring of predetermined sex can be produced in a variety of farm animal species. However, the relatively small number of sperm available for insemination imposes limitations. The IVF route in cattle has been shown to be practicable even though the use of sorted sperm leads to a reduction in the rate of embryo production. The effect of the dye on embryonic development is perhaps not surprising since a delay of embryo development *in vivo* and a reduction in litter size has been noted in rabbits and pigs after insemination with sorted sperm (4,10). Search for other dyes that might be used effectively for DNA analysis, but which might be more benign, has so far been unsuccessful.

The pregnancy rates obtained in initial trials, espe-

cially with frozen embryos of predetermined sex, were somewhat lower than might be hoped for, but this could no doubt be improved with further experience. For example, a pregnancy rate of around 50 percent has been achieved following direct transfer of frozen unsexed IVF embryos in commercial practice.

Intratubal surgical insemination is not a practical route for general application in any farm animal species. On the other hand, laparoscopic AI is used quite extensively for uterine insemination of frozen semen in sheep breeding. In addition, it has recently been shown that deep intrauterine insemination in cattle using very small numbers of sperm can lead to the establishment of pregnancies (15). Thus, this could be another route for practical application of sexed semen especially if the sorting rate could be increased appreciably (16).

In the majority of experiments fresh semen, or semen stored unfrozen for up to 24 hours, has been used for sorting, but frozen-thawed semen has also been used. IVF has generally been carried out within one or two hours of sorting, but sorted sperm can be stored for longer periods in suitable buffers at 5°C. This allows transfer to other locations for IVF or insemination (16). So far only preliminary experiments have been carried out on freezing sperm after sorting, but this option would extend still further the ability to apply the technology in a variety of breeding programs.

Conclusion

During the years that I have been associated with developments in artificial breeding technology, I have seen many advances, particularly in the scope of research, and to a lesser extent, in application. AI and frozen semen have been exceptional in the speed and extent to which they have been taken up in the commercial practice of animal breeding. This perhaps is due to the fact that the technologies produce results which are equivalent to those of natural mating and at a relatively low cost, yet they provide huge advantages both for management and for breed improvement. Embryo transfer is more costly than AI and results tend to be more variable, yet it has the major advantage of delivering the entire animal genome. This could become a very powerful technology indeed if it combines good genetics with the possibility of producing offspring of preselected sex. The route of *in vitro* embryo production involves the manipulation of many complicated biological processes associated with oocyte maturation, fertilization and embryonic development. All these are not yet fully understood and although the technology can already be applied on a limited scale where the economic advantages are acceptable, I am confident that further research will soon lead to greater efficiency and thus to a greater availability of use of sexed semen in animal breeding.

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