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Directional Freezing: Next-generation Technique of Semen Cryopreservation

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Introduction

Livestock sector plays a major role in the welfare of India's rural population. India has rich and diverse genetic resources with 37 well defined breeds of cattle and 13 registered breeds of buffaloes. Dairy cattle and buffalo production has undergone a major transformation resulting into a substantial increase in milk production thus helping our country to achieve top position in milk production in the world. Various animal breeding policies for selection and faster multiplication of genetically superior cattle and buffalo germplasm, have contributed significantly to increase the milk production but productivity is still very low. Therefore, there is a need to mitigate the gaps and further optimize the use of resources and undertake extensive field progeny testing programs for genetic improvement of cattle and buffaloes. Any progeny-testing programme begins with a decision on the number of bulls to be used under the test programme. The higher the number of bulls under test, higher will be the probability of getting bulls with high genetic potential. The technique of semen cryopreservation has enabled the initiation of large-scale breeding programs. They rely on the collection and cryopreservation of semen from a large number of selected test bulls while conducting field tests to evaluate the performance of their offspring. Often the tested male is no longer at the facility by the time the evaluation process is over. Currently, under field progeny testing programs, many semen freezing laboratories in the country are involved in cryopreservation of up to 40,000–50,000 insemination doses of each test bull and stored under liquid nitrogen which requires large space and high cost to maintain the germplasm. The field evaluation process takes about 4-5 years, at the end of which a decision is made whether to use the bull for future artificial inseminations (AI) or to discard the samples. Most of times, only 10% of the bulls are selected to be used for breeding at the end of progening testing, therefore, semen stored of rest of the bulls are disposed off. To solve these shortfall in existing protocol, directional freezing may become an option in which semen can be frozen in large volume and higher concentration in reusable containers to save space and costs and after evaluation of bulls, the selected semen can be thawed, diluted and

repackaged in straws and refrozen for genetic improvement programs. This method is also called double freezing.

What is double freezing method?

In the double freezing method, highly concentrated spermatozoa (about 450 × 10^6 per ml) is cooled down to 5°C and loaded into a large cryogenic tube and then the semen was frozen by a directional freezing technique. After directional freezing, the tubes are plunged into liquid nitrogen for bulk storage and only when needed (progeny testing is over), the tubes are passively warmed at room temperature, followed by thawing at 37° C for 30 s in a water bath. The thawed-semen is re-extended, loaded in mini straws and refrozen.

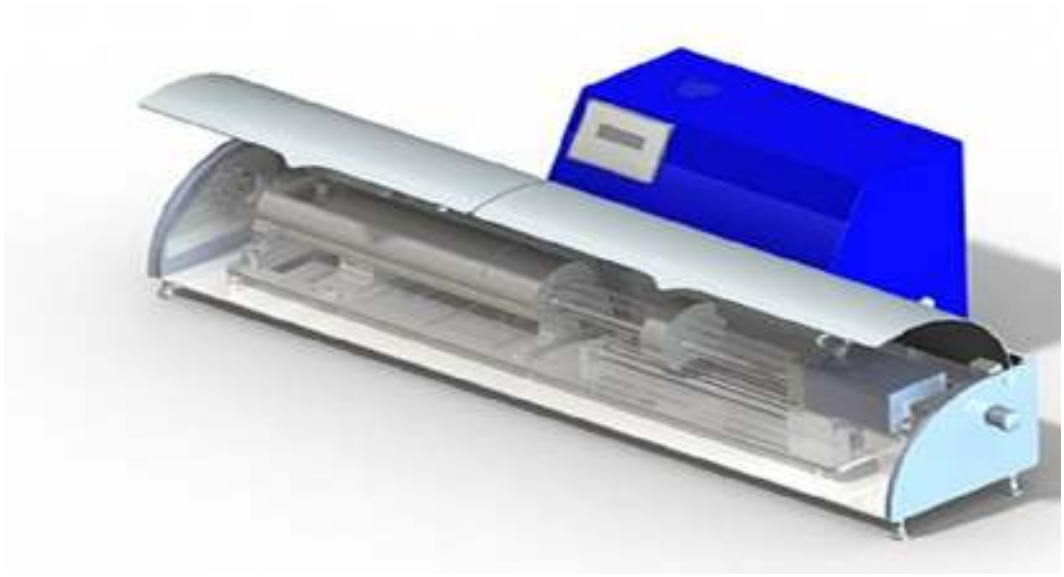

Figure: Multi Thermal Gradient (MTG) freezing systems.

What is directional freezing technique?

Directional freezing is based on a simple thermodynamic principle where ice crystals are precisely controlled through the sample by regulating the velocity of the sample movement through the predetermined temperature gradient which uses the Multi-Thermal Gradient device (Fig. 1) (MTG®; IMT Ltd., Ness Ziona, Israel; Harmony CryoCareTM, IMT Ltd., Ness Ziona, Israel). The MTG technology allows precise control over ice crystal propagation during the freezing process, thereby substantially reducing the mechanical damage caused to cells during freezing. Large volume freezing can be done by the directional freezing technique. The semen in the test tube is moved at a constant velocity (V) through a linear temperature gradient (G= 5.5° C/mm, from5 to -50°C) so that the cooling rate (G × V = 330° C/min)

and ice front propagation are precisely controlled. Seeding is done automatically for 100 s at -50° C. After the initial seeding stage, the semen sample is advanced at a constant velocity through a linear temperature gradient. After directional freezing, the tubes are plunged into liquid nitrogen.

Is directional freezing technique different from conventional freezing?

In general, the freezing rate must inhibit the formation of intracellular ice by allowing the diffusion of water out of the cell, while concurrently permitting extra-cellular ice formation at a size that minimizes mechanical damage to the cell. In conventional vapor freezing technology, it is not possible to control the velocity of ice-front propagation and the crystals disrupt and kill sperm. The directional freezing method employs multi-gradient heat transfer technology and allows precise control of the velocity and the morphology of the ice-front propagation. The directional freezing technology, although also apparently expensive, is more durable for movement and use outside the laboratory, is easy to use and requires little to no maintenance. The conventional freezing is suitable for small size of samples that are 5 milliliters or less in volume. This is partially due to the fact that, in large samples, some parts of the sample (usually the outer zone) may chill or warm faster than other parts. Thus, freezing and storage of semen is performed regularly using mini (¼ cc) or midi (½ cc) straws. But directional freezing technique is well suited for freezing large volume of semen keeping precise control over ice crystal propagation during the freezing process. In this technique, low glycerol concentration is required; therefore, protect sperm from toxicity of cryoprotectant. Reduced osmotic stress due to a faster freeze rate - tests have shown significant increases in hypo-osmotic resistance. To date, many studies have compared double freezing and the Liquid nitrogen vapour freezing technique. In many reports it was observed that increased post-thaw motility and a comparable number of successful pregnancies in cows after a double freezing/thawing cycle. One study reported no differences in total or progressive motility, functional plasma membrane integrity, acrosome integrity, mitochondrial status and DNA fragmentation in stallions before and after both freezing methods.

Advantages of double freezing technique

Semen sorting

The technique for sorting of sperm cells was developed about two decades ago. By the selective use of X-chromosome bearing spermatozoa for artificial insemination in endangered species, the number of females in the population can be enhanced resulting in population growth. Studies in a number of species showed already that this technique is suitable for use in nondomestic animals. These include mammals such as dolphin, rabbit, rhinoceros and elephant. In the dairy industry, this same technique is in extensive use as a mean to produce replacement cows and buffaloes for the herd. It was also

demonstrated in domestic mammals that semen can be frozen-thawed, sex-sorted and then refrozen-thawed and still maintain viability and fertilizing ability. One of the major technical obstacles in turning this into a widespread technique is the fact that the number of sperm sorting facilities around the world is only few. To get semen sorted collected at far distance from the facilities, double freezing is the only option. The problem can be overcome if semen can be frozen-thawed at the site of collection, sorted and then refrozen for AI. During the sorting process, dead or damaged cells are sorted out so the sample used for the second freezing is rich in viable, motile cells. Thus, double freezing may become handy under such circumstances.

Efficient breeding programs from progeny tested bulls

Breeding programs in domestic animals, primarily in the dairy industry, rely heavily on prolonged field progeny testing and at the end testing programs just a few breeding males are selected for future breeding. Various studies revealed there is no difference between the conventional and the double freezing methods when conception rate was evaluated in heifers and primiparous cows. These results suggest that breeding programs can aim at selecting bulls that, among their other traits, will also perform well following the double freezing process. The costs involved in maintaining frozen semen bank are huge but funds are often scarce. The costs involved in the collection and processing of semen from multiple bulls under evaluation in commercial insemination centers are enormous. An alternative procedure i.e. directional freezing may reduce maintenance costs, time and space for storing frozen semen by freezing of one or both ejaculates of each bull in one special test tube (12 mL) and only when needed (when the bull is a “proven bull”), the test tube will be thawed and will then be refrozen in regular mini straws. The use of large volume freezing has many advantages over that in small volumes (straws and pellet), for example, 10000 straws made from 25 ejaculates (400 straws/ejaculate). These 10000 straws will fit into 13 goblets (750 straws/goblet). In comparison, when freezing a large volume (12 mL test tube) the 25 ejaculates will be frozen in 25 test tubes which will be stored only in 2 goblets. This means that 6.5 times more goblets will be need by using straws in comparison to test tube freezing. In addition this method will save money in labour and consumables (printing, filling, sealing, LN_2 for freezing and for storage, etc.) required for freezing of semen.

Conservation of germplasm of endangered species

Apart from major farm animals, India has a remarkably wide variety of wild animals native to the country. It is home to tigers, lions, leopards, pythons, wolves, foxes, bears, crocodiles, rhinoceroses, camels, wild dogs, monkeys, snakes, antelope, deer, bison and Asian elephant. Declining natural habitats, human–animal conflicts and illegal poaching of endangered animals have lead to the extinction

of several wild animals. It is highly likely that in the next decades some of the wild animals will only exist in captivity. Since India is home to a number of rare and endangered animal species, wildlife management in the country is essential to preserve these species. Therefore if efforts are not immediately taken to establish self-sustaining captive populations, these animals will only exist as skeletons in the natural history museums. The use of assisted reproduction techniques(ART) and gamete preservation are imperative for establishing self-sustaining captive and natural habitat populations of these animals. The benefits of the double freezing technique for endangered species conservation are enormous. In wildlife conservationthis will open the way to the establishment of genome resource banks. Many times, the captive population of these animals is not self-sustaining with a limited number of breeding males and their genetic diversity and life span are declining day by day due to inbreeding. One way to overcome this is to import the animals from the wild. Directional freezing will be a more sustainable alternative method of breeding without removing them from their natural habitat.

Whole Ovary Freezing

In addition to semen cryopreservation, from many years, cryopreservation of large organs has been attempted with limited success because of the problems associated with heat transfer and irregular rate of cooling between the core and the periphery of the organ. To overcome these problems, two approaches have been suggested: the vitrification and the directional freezing. Vitrification has many drawbacks such as chemical toxicity and osmotic shock following exposure to high concentration of cryoprotectants. Fractures of the organ could be caused by during vitrification and thawing. By this method, successfully whole ovaries have been cryopreserved and transplanted in many species. Cryopreservation of intact ovaries with their vascularity confirming that wholeorgan ovary transplantation and use of follicles for ART may be a viable option inthe future.

Conclusions

The directional freezing could be very useful for field progeny testing programs, conservation of germplasm of endangered species and whole ovary cryopreservation because the ability of this technique to control the ice crystal morphology, the homogeneous cooling rate through a large volume sample and the continual seeding during the whole freezing process. Cryopreservation of whole ejaculates of bull semen has many folds benefits compared to freezing by conventional freezing and save many expenses related to freezing and storage.

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