Processing techniques for canine semen

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Introduction

The use of artificial insemination with either cooled or frozen canine semen continues to gain in popularity world-wide. Processing and extension of canine semen is typically straightforward; however, sperm selection methods more commonly used in other species can be adapted for use with canine sperm. Processing after collection can be used to remove seminal plasma and prostatic fluid, to increase the concentration of the ejaculate, and to preferentially select normal sperm. Supplies and materials that may be helpful include a variable speed centrifuge, preferably with hanging buckets, plastic or glass 15mL conical tubes, disposable pipettes, flat-bevel needles, and larger-volume (1 mL to 5 mL) micropipettors and disposable tips.

Extension

A variety of commercially prepared semen extenders are available for use specifically with canine semen. Cooling and freezing media can also be prepared within the laboratory, but this requires the practitioner to assume all responsibility for quality control of raw materials and final product composition (pH, osmolality, bacterial contamination, etc). Given the ease of procurement, high quality, and low costs of commercially prepared extenders, this is likely a more practical option for practitioners.

Semen may be extended for the purposes of cooled storage/transport or cryopreservation. This process involves dilution of semen, and then cooling and storage at appropriate temperatures until insemination. Cooled semen has several advantages over frozen semen, namely its procedural ease, no need for specialized equipment for preparation or use,[1] and possibly fewer restrictions on transport/importation.[2] Extenders for cooling typically contain fructose or glucose, and fructose-containing extenders may result in longer sperm longevity.[2] Extended semen should be stored at 4-12°C; temperatures below 4°C may result in reduced sperm longevity. When stored at an appropriate temperature, sperm longevity up to 23 days has been reported,[2] but prolonged storage is typically impractical and unnecessary and results in decreased physical and functional characteristics.

Extension for cryopreservation can be performed with single-step or two-step protocols.[3] Ideally, the sperm rich fraction should be isolated with minimal collection of prostatic fluid. Centrifugation is performed to concentrate the sperm and remove excess prostatic fluid; sperm washing can be performed as an initial step, based on clinician preference and the specific protocol used. Specific canine freezing media can be purchased, or human sperm cryopreservation media can be used. The most common cryoprotectant in canine freezing media is glycerol, and glycerol appears to outperform amide cryoprotectants for canine sperm.[4,5] Although most freezing media used for canine sperm contains egg yolk, soy bean based extender has been evaluated for use in this species.[6] Although not in common use, the soy based extenders have a distinct advantage of being free from animal proteins which may be beneficial when frozen semen is subjected to international transport.

Sperm selection methods

A wide variety of sperm selection methods are currently available, including sperm washing,7,8 swim up,[9,10] density-gradient centrifugation,[11-13] single-layer centrifugation,[8,14-16] and glass wool filtration.[11] These methods vary greatly in the recovery rate and sperm quality assessment parameters such as motility, morphology, chromatin integrity, viability and acrosome integrity.[17]

Sperm washing

Washing of sperm can be achieved by diluting the ejaculate with extender, and centrifuging the sample in order to separate the seminal plasma from sperm. The supernatant can be manually removed with a disposable 3mL pipette or needle and syringe. Selection of centrifugation speed needs to balance reduction in sperm loss while mitigating damage to sperm. Higher centrifugation speeds improve sperm

recovery and produce a more tightly packed pellet. However, higher centrifugation speeds are also associated with increased dead/moribund sperm.[7] The pellet produced contains dead, moribund, and abnormal spermatozoa as well as viable spermatozoa.[8] Recommended centrifugation speeds range from 700-900g for 5-10 minutes. Setting the centrifuge deceleration speed to the lowest setting will result in less turbulence to disrupt the sperm pellet during braking.

Swim up

Swim-up is a method of purification most commonly used for selection of sperm for procedures such as in-vitro fertilization, and is considered a basic manipulation for this procedure. In a standard swim up procedure, the sperm are washed, and the resultant pellet is over layered with medium and incubated at 37°C in 5% CO_2.[18] After incubation, the supernatant containing actively mobile sperm is removed. Modification of this technique may reduce sperm lost during the centrifugation phase.[8] Briefly, a small volume of ejaculate is placed under a layer of medium; semen samples are then incubated in a vertical position, and the top layer of medium is removed and re-suspended, allowing collection of sperm that have "swam up".[8] Most viable sperm are recovered after an *in vitro* incubation step of 15 minutes; as the time of incubation increases, so does the number of degenerated or damaged cells.[9]

Density-gradient centrifugation

Density-gradient centrifugation separates normal from abnormal spermatozoa based on isopyknic point; sperm are centrifuged through layers of colloid. After removal of the supernatant containing the colloid layers and discarded sperm, the subsequent pellet is re-suspended in washing medium and centrifuged again to remove any residual colloid prior to insemination or freezing. This technique results in selection of a sperm population that demonstrates improved quality (ie, higher motility, viability, and acromsome integrity).[12] Density-gradient centrifugation has also been used to successfully separate erythrocytes from sperm.[13] The main drawbacks of the technique are related to technical competency; formation of the colloid layers is integral to successful separation, which requires some practice on behalf of the user to master. Preparation of the conical tubes can be time consuming, particularly if large volumes need to be prepared. Centrifugation speeds are typically slower with an increased centrifugation time, for example, 20 min at 300 *x g*.

Single-layer centrifugation

This technique is similar to density-gradient centrifugation, but is simplified in that it only uses one layer of colloid. From a practical point of view, this results saving time and effort in preparation of the sample whilst still selecting a normal highly motile sperm population.[16] Current research regarding the separation of other cell types from single-layer centrifugation is lacking.

Glass wool filtration

Glass wool filtration is easily performed in practice, with no specialized equipment needed. Glass wool is packed to a depth of 6mm in a 1-mL disposable syringe barrel (ie, tuberculin syringe). The column is rinsed 2-3 times with 1mL of medium to remove loose glass wool fibers and other particles prior to use. The end of the syringe barrel is placed in a 1.5mL Eppendorf tube, perforated at the lid to accommodate the tip of the syringe.[11] Additional holes are punched in the tube to equilibrate pressure, and the tube and column can then be placed in a rack in the incubator. Glass wool filtration can be used to remove cryo-damaged sperm after freezing-thawing,[11] and this technique is thought to trap mechanically-damaged spermatozoa.

Sex sorting of canine semen

Sex-sorted semen has become common in cattle reproduction, but is not commonly practiced or commercially available for canine sperm at this time. However, this technology has been evaluated under field conditions for the purposes of research.[19] Conception rates in both the X- and Y- sorted sperm

groups were lower than unsorted control groups (25% and 20% vs. 55%, respectively), but both sorted groups produced approximately 86% of the puppies being born the desired sex.

Conclusions

A variety of processing techniques are available for canine semen; these techniques are commonly used in other species and can be adopted in the laboratory with minimal investment in additional equipment. Although these techniques can be successfully used to select normal sperm from abnormal, removal of other cell types (i.e., red blood cells or leukoycytes) presents a significant clinical advantage for processing canine semen. Further research and improvements on these techniques may improve success rates with assisted reproductive technologies in canids.

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