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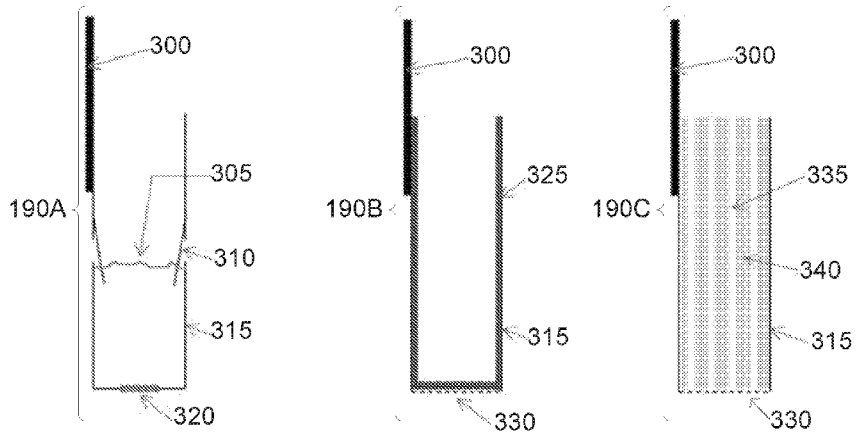
FIG. 3



(57) Abstract: A canister for liquid cryogen storage comprises a cylindrical cup with a circular side wall and a bottom wall, a series of apertures in the circular side wall, and a flange affixed to an interior surface of the circular side wall at a position adjacent to the apertures.

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Containers for Liquid Nitrogen Storage of Semen Straws

All subject matter of the Priority Application(s) is incorporated herein by reference to the extent such subject matter is not inconsistent herewith.

SUMMARY

In some embodiments, a canister for liquid cryogen storage includes: a cylindrical cup with a circular side wall and a bottom wall; a series of apertures in the circular side wall; and a flange affixed to an interior surface of the circular side wall at a position adjacent to the apertures.

In some embodiments, a canister for liquid cryogen storage includes: a cylindrical cup with a circular side wall and a bottom wall; and a layer of absorbent material affixed to the interior surface of the circular side wall, the absorbent material absorbent to a liquid cryogen.

In some embodiments, a canister for liquid cryogen storage includes: a cylindrical cup with a circular side wall and a bottom wall; and a solid mass of a size and shape to substantially fill the interior of the cylindrical cup, the solid mass including a plurality of cavities positioned vertically in the solid mass.

The foregoing summary is illustrative only and is not intended to be in any way limiting. In addition to the illustrative aspects, embodiments, and features described above, further aspects, embodiments, and features will become apparent by reference to the drawings and the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 depicts aspects of a Dewar.

FIG. 2A shows a cross section of a Dewar.

FIG. 2B illustrates recommended practices with a Dewar.

FIG. 2C depicts poor handling practices with a Dewar.

FIG. 3 is a schematic of three canister embodiments.

FIG. 4A is a cross-section depiction of a canister.

FIG. 4B is a cross-section depiction of a canister.

FIG. 4C is a cross-section depiction of a canister.

FIG. 4D is a cross-section depiction of a canister.

FIG. 5 is a graph depicting test results.

FIG. 6 is a graph depicting test results.

FIG. 7 is a graph depicting test results.

FIG. 8 is a graph depicting test results.

FIG. 9 is a graph depicting test results.

FIG. 10 is a graph depicting test results.

FIG. 11A is a graph depicting test results.

FIG. 11B is a graph depicting test results.

FIG. 11C is a graph depicting test results.

FIG. 11D is a graph depicting test results.

FIG. 12A is a graph depicting test results.

FIG. 12B is a graph depicting test results.

FIG. 13 is a graph depicting test results.

DETAILED DESCRIPTION

In the following detailed description, reference is made to the accompanying drawings, which form a part hereof. In the drawings, similar symbols typically identify similar components, unless context dictates otherwise. The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. Other embodiments may be utilized, and other changes may be made, without departing from the spirit or scope of the subject matter presented here.

Artificial insemination of dairy cattle is a common practice in the developing world that can improve farmer incomes and food security. Maintaining the fertilizing potential of frozen semen as it is manipulated, transported and stored is crucial to the success of this process. Described herein are technological improvements to protect semen from inadvertent thermal fluctuations that occur when poor handling practices are used with standard equipment. When frozen semen is subjected to poor handling practices using standard equipment, certain characteristics of semen biology associated with fertility are negatively affected. Herein is described several design improvements and results from thermal performance tests of several improved prototypes. Experiments have compared semen that has experienced poor handling practices in standard and improved equipment.

These data suggest that an improved canister can better maintain the fertility of frozen semen when subjected to poor handling.

Over the last few centuries selective breeding has developed dairy cattle breeds that produce large quantities of milk. By contrast, indigenous cows in the developing world are far less productive (N. Makoni, T. Redda, J. van der Lee, R. Mwai, and A. van der Zijpp, "White gold - Opportunities for dairy sector development collaboration in East Africa," 2014 and A. K. Kahi and T. O. Rewe, "Biotechnology in livestock production : Overview of possibilities for Africa," African J. Biotechnol., Vol. 7, No. 25, pp. 4984-4991, 2008, which are each incorporated by reference). Recognizing this large discrepancy, many developing counties strive to improve milk yields—and therefore farmers' incomes and food security—by utilizing the genetics of nonendemic breeds. While traditional mating of imported cattle is used to generate both cross and full breeds, artificial insemination (AI) is the prevailing method used to accomplish this goal (E. van Marle-Köster and E. Webb, "Current and Future Reproductive Technologies and World Food Production," in Current and Future Reproductive Technologies and World Food Production SE - 10, Vol. 752, G. C. Lamb and N. DiLorenzo, Eds. New York, NY: Springer New York, 2014, pp. 199-211, which is incorporated by reference).

Perhaps the greatest breakthrough to impact the practice of AI came in the 1950s when glycerol was discovered to act as a cryoprotectant for dairy bull semen (R. W. Bratton, R. H. Foote, and J. C. Cruthers, "Preliminary Fertility Results with Frozen Bovine Spermatozoa," J. Dairy Sci., Vol. 38, No. 1, pp. 40-46, 1955 and C. Polge and L. E. A. Rowson, "Fertilizing capacity of bull spermatozoa after freezing at -79 C," Nature, Vol. 169, pp. 626-627, 1952, which are each incorporated by reference). This advance allowed for a much greater temporal, and therefore spatial, separation between semen collection and insemination. Now, semen is generally collected, packaged into semen straws, and frozen at centralized facilities. The frozen semen is then shipped throughout the world in Dewars containing liquid nitrogen (LN).

Four variables affect whether AI will result in conception: heat detection, inseminator efficiency, fertility of the heifer, and the fertility of the semen (C. Polge and L. E. A. Rowson, "Fertilizing capacity of bull spermatozoa after freezing at -79 C," Nature, Vol. 169, pp. 626-627, 1952 and K. B. Desta, "Analyses of Dairy Cattle -Breeding Practices in Selected Areas of Ethiopia Dissertation," 2002, which are each incorporated by reference). Mammals are fertile only for a limited window of time during each

reproductive cycle, so effectively determining that a heifer is in heat is vital for AI success. Similarly, the competency of an inseminator is important to properly deliver semen hygienically and on target. These first two variables are most effectively managed through proper AI technician training. Heifer fertility, the third variable influencing AI success, is affected by diseases such as mastitis, the length of the post-partum waiting period, and nutrition, and is shaped by heifer management throughout its life.

Semen fertility, the final variable in AI success, is most commonly affected by handling subsequent to semen packaging and freezing (B. W. Pickett, "Factors affecting the utilization of frozen bovine semen for maximum reproductive efficiency," A.I. Dig., Vol. 19, No. 2, p. 8, 1971 which is incorporated by reference). A vast amount of research has optimized the number of sperm packed per straw, the additives that supplement semen, and the precise protocols used to properly freeze this mixture (E. M. Walters, J. D. Benson, E. J. Woods, and J. K. Critser, "The history of sperm cryopreservation," 2009 and W. V Holt, "Basic aspects of frozen storage of semen," Anim. Reprod. Sci., Vol. 62, No. 1-3, pp. 3-22, Aug. 2000, which are each incorporated by reference). Once a lot of semen has been frozen into individual straws, bull stud ranches generally perform quality control assessments by assaying in vitro characteristics that are associated with fertilizing potential (H. Rodriguez,-Martinez, "Can We Increase the Estimative Value of Semen Assessment," Reproduction Domest. Anim., Vol. 41, pp. 2-10, 2006 and L. Z. Oliveira, F. M. Monteiro, E. Carla, and C. Celeghini, Success in Artificial Insemination - Quality of Semen and Diagnostics Employed, 2013, which are each incorporated by reference). Consequently, semen sold by most reputable bull stud ranches is generally highly fertile, and if stored properly in LN, will retain its fertilizing potential indefinitely (R. H. Foote, "The history of artificial insemination : Selected notes and notables," J. Anim Sci., Vol. 80, pp. 1-10, 2002, which is incorporated by reference).

However, subsequent improper handling by prematurely breaking the cold chain can dramatically decrease a straw's fertility. In order to prevent this thermally-induced semen damage, most developed world bovine inseminators are taught to never remove semen straws from LN for more than very brief periods, unless the straw is being thawed for immediate usage. Rules of thumb limit exposures to eight, five or even three seconds (B. Stroud, "Consequences of mishandling frozen semen and embryos," Proceedings, Appl. Reprod. Strateg. Beef Cattle, pp. 191-204, 2012, which is incorporated by reference). These rules are stressed during inseminator training because very short

exposures to ambient temperatures can cause large temperature fluctuations within the straws (W. E. Berndtson, B. W. Pickett, C. D. Rugg, and F. Collins, "Procedures for Field Handling of Bovine Semen in Plastic Straws," pp. 51-60, 1973, which is incorporated by reference). While these fluctuations are often not large enough to thaw the straw's contents, they do cause cumulative and irreversible damage that negatively impacts the fertilizing potential of semen (W. V Holt, "Basic aspects of frozen storage of semen," Anim. Reprod. Sci., Vol. 62, No. 1-3, pp. 3-22, Aug. 2000; B. Stroud, "Consequences of mishandling frozen semen and embryos," Proceedings, Appl. Reprod. Strateg. Beef Cattle, pp. 191-204, 2012; B. W. Pickett, W. E. Berndtson, and J. J. Sullivan, "Influence of seminal additives and packaging systems on fertility of frozen bovine spermatozoa," J. Anim. Sci., Vol. 47, Suppl 2. pp. 12-47, 1978; O. Rodriguez, W. Berndtson, B. Ennen, and B. Pickett, "Effect of rates of freezing, thawing and level of glycerol on the survival of bovine spermatozoa in straws," J. Anim. Sci., pp. 129-136, 1975 and B. W. Pickett, "Factors Affecting the Utilization of Frozen Bovine Semen for Maximum Reproductive Efficiency," First N.A.A.B. Tech. Conf., p. 64, 1966, which are each incorporated by reference).

Much of the equipment used to store and categorize straws in a Dewar was designed to promote proper handling practices. The plastic goblets and cane assemblies used in Dewars with 20 liter or greater capacity have been demonstrated to help improve protection from inadvertent exposure (W. E. Berndtson, B. W. Pickett, C. D. Rugg, and F. Collins, "Procedures for Field Handling of Bovine Semen in Plastic Straws," pp. 51-60, 1973, which is incorporated by reference). These larger Dewars, however, are used less frequently in developing countries where farms are typically much smaller, increasing the need for an AI technician to be mobile. In these settings smaller 3 L Dewars are most common as they are easily transported by motorcycle (P. Barua, "Analytical Documentation of the Artificial Insemination Programme of BRAC," BRAC Res. Rep., 2006, which is incorporated by reference). The smaller size of these Dewars does not allow the use of the canes. Consequently goblets are uncommon and straws are often placed directly in the canister.

A limited number of studies and a large amount of anecdotal evidence suggest that poor handling of frozen semen is a frequent occurrence. One study compared several morphological and motility characteristics in semen stored at operational farms and a facility where proper handling practices were tightly controlled. Semen stored at the farm

showed significant damage, suggesting that proper handling guidelines were not followed (M. Pace, L. Peterson, G. VanDellen, E. Waterman, R. Robbins, and J. Sullivan, "Effect of field working storage upon quality of frozen bull spermatozoa packaged in 0.5 ml ampoules and 0.5 ml French straws," in American Society of Animal Science Annual Meeting, 1977, p. 194, which is incorporated by reference). A more recent study measured in vitro fertilization rates and showed that if post-thaw sperm microscopically diagnosed as poor quality—attributed by the author as largely due to routine poor handling practices—are removed, there is a 9% improvement in embryo fertilization (B. Stroud, "Consequences of mishandling frozen semen and embryos," Proceedings, Appl. Reprod. Strateg. Beef Cattle, pp. 191-204, 2012 and F. N. Schrick, F. M. Saxton, and B. K. Stroud, "Assessment of semen quality for predicting recovery of viable embryos of superovulated cattle," in Joint Conv Proc CETA/AETA, 2003, which are each incorporated by reference).

Proper technique dictates that canisters be held below the frost line in the neck of the Dewars for limited amount of time. However, it has been demonstrated that sometimes canisters are raised well above the frost line in the Dewar neck or removed entirely from the Dewar neck.

Figure 1 depicts a representative example of a commercially-available Dewar (*e.g.* YDS -3 3 L Dewar, Chart Industries, Garfield Heights, OH, and/or XTL3 3L Dewar, Taylor-Wharton Industries, Mobile AL). A locking cover 100 reversibly covers the top opening of the container. A cork 150 reversibly mates with the interior of the neck tube 120. A handle 160 is affixed to the exterior of the container. An evacuating nozzle 110 is positioned at the upper face of the container. The neck tube 120 provides a conduit between the outer shell 140 and the inner vessel 180. Multi-layer thermal insulation 170 is positioned between the outer shell 140 and the inner vessel 180 and surrounding the neck tube 120. Adsorbent material 130 is positioned at the upper surface of the inner vessel 180, the adsorbent material surrounding the neck tube 120. Multiple canisters 190 are positioned in the lower portion of the inner vessel 180. Each canister 190 includes a handle that projects upward and into the neck tube 120.

Training is often less rigorous in the developing world, implying that handling-induced semen damage is likely an even greater problem in these areas. Indeed, the number of AI services per conception is generally higher in developing countries (E. van Marle-Köster and E. Webb, "Current and Future Reproductive Technologies and World

Food Production," in Current and Future Reproductive Technologies and World Food Production SE - 10, Vol. 752, G. C. Lamb and N. DiLorenzo, Eds. New York, NY: Springer New York, 2014, pp. 199-211, which is incorporated by reference), and improper semen handling contributes to these differences (T. Woldu, "Factors affecting conception rate in artificially inseminated cattle under farmers condition in Ethiopia," J. Cell Anim. Biol., Vol. 5, No. 16, pp. 334-338, 2011, F. M. Kivaria, J. P. T. M. Noordhuizen, and A. M. Kapaga, "Prospects and constraints of smallholder dairy husbandry in the Dar es Salaam region, Tanzania," Outlook Agric., Vol. 35, No. 3, pp. 209-215, 2006 and A. L. Alejandrino, C. O. Asaad, B. Malabayabas, A. C. De Vera, M. S. Herrera, C. C. Deocaris, L. M. Ignacio, and L. P. Palo, "Constraints on dairy cattle productivity at the smallholder level in the Philippines," Prev. Vet. Med., Vol. 38, No. 2-3, pp. 167-178, 1999, which are each incorporated by reference).

Figures 2A, 2B and 2C depict aspects of handling of semen straws within a Dewar. Figure 2A depicts a cross-section view of a Dewar including a cover 100 and a neck tube 120. The device is positioned for storage of the semen straws within the storage region of the inner vessel 180. The neck tube 120 is affixed at its lower edge to an inner vessel 180. An outer shell 140 surrounds the inner vessel 180. Multiple storage canisters 190 are held within the inner vessel 180 for storage. During use, the inner vessel 180 would include a cryogen, such as liquid nitrogen (LN). Figure 2B shows a cross-section view of the Dewar to illustrate recommended practices to access semen straws within a canister. The canister is brought up to the edge of the frost line 200 for access, but does not pass beyond the frost line 200. Figure 2C depicts a cross-section view of a Dewar to illustrate common poor handling practices. The canister is elevated above the frost line 200 of the Dewar while semen straws are removed.

Generally multiple semen straws are stored within a canister. If poor handling practices are routinely used—such as each time an AI technician removes a straw for immediate usage—straws within the inventory will experience multiple thermal exposures. It has been suggested that semen damage results when the bulk product temperature rises above -130° C, the glass transition temperature of water, and then cools back down when re-introduced to LN (B. Stroud, "Consequences of mishandling frozen semen and embryos," Proceedings, Appl. Reprod. Strateg. Beef Cattle, pp. 191-204, 2012 and W. E. Berndtson, B. W. Pickett, C. D. Rugg, and F. Collins, "Procedures for Field

Handling of Bovine Semen in Plastic Straws," pp. 51-60, 1976, which are each incorporated by reference).

In an effort to improve AI success rates in the developing world, inexpensive and easy to use technology has been developed that protects semen from thermal exposures, as described further below. Aspects of semen damage that occur when frozen straws are subjected to common poor handling practices were quantified. The design and thermal performance of three semen canister prototypes is described. Frozen semen stored in a standard and in an improved canister were subjected to repeated ambient temperature exposures. Subsequent analysis shows that semen stored in the improved canister possesses characteristics of more highly fertile semen.

Prototype Design and Testing

The canister found in most small portable Dewars used for AI consists of a cylindrical cup with a handle to allow manipulation inside a Dewar and to rest on a hook at the neck. A grate is attached to the canister base to allow LN to flow out as it is raised.

Three prototype canister designs were developed and evaluated (see Figure 3). Figure 3 is a cross-section schematic of Prototype Design 1 190A (left), prototype Design 2 190B (center), and prototype Design 3 190C (right).

Figure 3 shows prototype Design 1 190A to the left side of the Figure. The embodiment includes a handle 300 affixed to the canister wall 315. Design 1 190A is shown with a cryogen 305, such as liquid nitrogen, within the canister, while a vapor guard 310 maintains the liquid nitrogen 305 within the canister. A porous or permeable membrane 320 is positioned at the lower surface of the canister.

The prototyped canister Design 1 190A retains a liquid cryogen, such as LN, during regular operation resulting in lower semen temperatures during poor handling practices (see Figure 3). In the embodiment illustrated in Figure 3, Design 1 190A includes a sealed canister base to retain LN when the canister is raised. The design features of design 1 also include drain holes, drilled along the circumference, anywhere from one quarter to halfway up from the bottom of the canister, the drain holes spaced to control the level of liquid cryogen 305 within the canister. The holes are positioned to allow the canister to fill when the liquid cryogen 305 level in the Dewar is below the top of the canister. With this design, a canister of Design 1 190A will gradually sink when inserted into the Dewar as the cryogen fills the canister.

Features of Design 1 include a vapor guard that is fixed to the inside wall of the canister along the circumference to cover the drain holes and extend below the cryogen liquid level. The function of the vapor guard skirt structure is to minimize cryogen vapor from flowing out of the holes when a canister of Design 1 is withdrawn from the Dewar during use.

In some embodiments, Design 1 includes a base fitted with a permeable membrane designed to allow a liquid cryogen to slowly fill over several hours while restricting any meaningful amount of the liquid cryogen from exiting during a straw extraction event lasting up to a few minutes. For example, in some embodiments the permeable membrane includes a sintered metal. The use of such a membrane will extend the effectiveness of this device when the liquid cryogen level in the Dewar drops below the elevation of the drain holes.

Figure 3 depicts prototype Design 2 190B in the center of the Figure. The embodiment includes a handle 300 affixed to the canister wall 315. A liquid cryogen absorbent material 325 is positioned adjacent to the inner surface of the canister wall 315. For example, in some embodiments the absorbent material is absorbent of liquid nitrogen. The embodiment 190B includes a grated base 330 at the lower face of the canister.

The canister wall and base are lined with an absorbent material positioned to absorb liquid cryogen and provide additional insulation (see Figure 3). The design permits the liquid cryogen to drain from a grated canister base as it is raised. Some embodiments include a LN absorbent material. Some embodiments include a LN absorbent material that includes a flexible aerogel. Some embodiments include a LN absorbent material that includes a woven fiberglass material. The LN absorbent material provides thermal isolation from the environment and blankets straws with cold nitrogen vapor as the liquid vaporizes. The thickness of the material is designed to store sufficient liquid cryogen for a short exposure up to several minutes. In some embodiments, the absorbent material is between approximately 2 mm and approximately 7 mm in thickness. The design including liquid cryogen absorbent material at the canister base allows liquid to fill and drain during use but minimizes natural convection.

Figure 3 illustrates prototype Design 3 190C at the right of the Figure. The embodiment includes a handle 300 affixed to the canister wall 315. The embodiment 190C includes a grated base 330 at the lower face of the canister. The embodiment 190C includes a solid mass 340 positioned within the canister. A series of apertures 335 are

positioned substantially vertically within the solid mass 340. The apertures 335 include an opening at the top face of the solid mass 340. The apertures 335 project vertically from the top face of the solid mass 340 into the solid mass 340 for a depth at least as long as a semen straw expected to be stored within the canister 315.

The canister is designed to include solid mass with a system of holes along its length to insert several straws (see Figure 3). The holes form apertures oriented along the long axis of the Design 3 canister. The holes can be designed to sort straws from different bulls. For example, in some embodiments the holes are sized, shaped, and positioned to store semen straws in different sections that can be easily identified by a user based on one or more of these factors.

The solid mass can be fabricated, for example, from a heat-conductive material, such as a thermally-conductive metal. In some embodiments, the solid mass of the canister is fabricated from aluminum. In some embodiments, the solid mass of the canister is fabricated from copper. In some embodiments, the solid mass of the canister is fabricated from stainless steel. The solid construction is designed to provide additional thermal mass relative to a standard canister, thus slowing the temperature rise during an exposure to remain below -130 degrees C.

We conceived of three approaches to improve the canister design to decrease thermal fluctuations within semen straws when the canister is removed from the Dewar. Prototype versions of these designs are depicted in Figure 4B, 4C and 4D.

Figure 4 depicts additional aspects of canisters for storage of semen straws within a Dewar. Figure 4A, 4B, 4C, 4D depicts prototype schematics in cross-section. Figure 4B, 4C and 4D depict aspects of Design 1, Design 2 and Design 3 as described above.

Figure 4A shows a depiction of a canister found in many small portable Dewars. Figure 4A depicts a representative example of the standard canister design in a cross-section view. The cylindrical cup 315 that holds semen straws is attached to a handle 300 that allows manipulation inside a Dewar and to rest on a hook at the neck. A grate 330 or drain holes are incorporated into the canister base to allow liquid cryogen to empty as it is raised. The canisters found in most small portable Dewars used for AI consist of a cylindrical cup with a handle to allow manipulation inside a Dewar and to rest on a hook at the neck. A grate or drain holes 330 are incorporated to the canister base to allow liquid cryogen to flow out as it is raised. A handle 300 is affixed to a top edge of the canister wall 315. The bottom of the canister includes a grated base 330.

Figure 4B depicts aspects of Design 1. When the canister is in the resting position in the Dewar, liquid cryogen enters the canister through drain holes 400. The drain holes 400 are positioned above the base of the container at a position approximately one third to one half of the length of the container. When the canister is raised, cold cryogen vapor (e.g. cold nitrogen vapor) fills the top half of the canister as liquid cryogen maintained at the base of the canister boils. A vapor guard 310 is included to minimize vapor exit via the drain holes. The vapor guard 310 is formed as a skirt or flap structure affixed at its upper edge to a position above the drain holes 400, with the lower edge of the vapor guard 310 extending below the level of the drain holes 400. In some embodiments, the lower edge of the vapor guard fully covers one or more of the drain holes. In some embodiments, the lower edge of the vapor guard partially covers one or more of the drain holes. Depending on the embodiment, a vapor guard can be fabricated from a thin metal sheet, for example fabricated from a stainless steel or aluminum sheet. In some embodiments, the vapor guard is fabricated from the same material as the canister walls.

The grate at the base of the canister is replaced with a solid metal surface 420 to prevent liquid cryogen draining. To accommodate low levels of liquid cryogen in a Dewar, the canister is fitted with sintered metal that acts as a permeable membrane to allow liquid cryogen to slowly fill over several hours 320.

Figure 4C depicts aspects of Design 2. This canister is lined with a liquid cryogen absorbent material 325 to both provide thermal insulation from the environment and blanket semen straws with cold cryogen vapor as the liquid vaporizes. In some embodiments, the absorbent material is a material that absorbs liquid nitrogen. In some embodiments, the absorbent material is a felted material, such as fabricated from cotton or polyester. In some embodiments, the absorbent material is an acrylic felt material. In some embodiments, the absorbent material is a fiberglass mesh or weave material. In some embodiments, the absorbent material is an aerogel material. In some embodiments, the absorbent material is a cryogel material. In some embodiments, the absorbent material is a cryogen-permeable material with porosity. The absorbent material can be, for example, between approximately 2 mm in thickness and approximately 7 mm in thickness, depending on the material used. The absorbent material can be, for example, approximately 2 mm in thickness. The absorbent material can be, for example, approximately 3 mm in thickness. The absorbent material can be, for example, approximately 4 mm in thickness. The absorbent material can be, for example,

approximately 5 mm in thickness. The absorbent material can be, for example, approximately 6 mm in thickness. The absorbent material can be, for example, approximately 7 mm in thickness. A grated metal surface 430 is included as a permeable layer and positioned to protect the liquid cryogen absorbent material.

Figure 4C depicts aspects of Design 3. A solid cylinder 340 is designed to fit snugly within the canister to increase thermal mass. In some embodiments, the solid cylinder is aluminum. A system of holes 335 is drilled along its length to accommodate semen straws. The holes 335 can be sized and positioned to contain semen straws in an easy position for handling and/or organization during storage.

In the first approach (Design 1) we sought to maintain a level of liquid cryogen within the canister by replacing the grate at the canister base with a solid metal surface 420 (*see* Figure 4B). We designed a canister that possesses drain holes 400 drilled along the circumference of the canister approximately half way (*e.g.* 5 cm) from its bottom to allow liquid cryogen to flow between the canister and the Dewar. In addition, the drain holes allow the canister to gradually sink when it is inserted into the Dewar. A vapor guard 310 is positioned adjacent to the drain holes 400, the vapor guard 310 affixed to the interior of the canister wall 315 at a position above the drain holes 400. This prototype is similar to several existing canisters with the notable difference that our design allows for significantly higher levels of liquid cryogen in the canister.

The intention of the features of Design 1 was to drive cold cryogen vapor over the top portion of the straws that are not submerged in liquid cryogen. However, when we removed our canister prototype from the Dewar neck, we noticed that a significant portion of cryogen vapor escapes from the drain holes. To minimize this effect we modified the design to include a vapor guard on the inside wall of the canister along the circumference that covers the drain holes and extends below the liquid cryogen level (*see* Figure 4B).

To accommodate low levels of liquid cryogen in a Dewar we fitted the canister with sintered metal 320 within the base that acts as a permeable membrane to allow liquid cryogen to slowly fill over several hours while restricting the loss of liquid cryogen during a straw extraction event lasting up to a few minutes (*see* Figure 4B). The use of this prototype extends the effectiveness of this device when the liquid cryogen level in the Dewar drops below the elevation of the drain holes.

In the second approach (Design 2) we sought to provide thermal insulation from the environment and blanket semen straws with cold cryogen vapor as liquid cryogen

vaporizes. To do this, we designed a canister lined with an liquid cryogen absorbent material (*see* Figure 4C). The thickness of the material was intended to store sufficient liquid cryogen for an exposure lasting several minutes. For canisters with a grated base, we also included the liquid cryogen absorbent material at the canister base to allow liquid to fill and drain during use but minimize natural convection of cold vapors when the canister was raised in the Dewar.

In the final approach (Design 3) we sought to increase thermal mass of the canister to slow the temperature rise during an exposure (*see* Figure 4D). We machined a solid aluminum cylinder 340 to fit snugly within the canister and drilled a system of holes 335 along its length to accommodate several semen straws. This prototype increases organization within the canister and allows straws from different bulls to be separated in different regions with a canister in a similar manner to how goblets function in larger Dewars.

In some embodiments, a canister for liquid cryogen storage includes: a cylindrical cup with a circular side wall and a bottom wall; a series of apertures in the circular side wall; and a flange affixed to an interior surface of the circular side wall at a position adjacent to the apertures. In some embodiments, the cylindrical cup includes: a cylindrical cup of a size and shape to fit through the neck of a Dewar and to be retained within the inner storage region of the Dewar. In some embodiments, the cylindrical cup includes: a cylindrical cup of a size and shape to retain a plurality of bovine semen straws.

In some embodiments, a canister for liquid cryogen storage includes a series of apertures in a circular side wall, wherein the series of apertures are positioned around the circular side wall at positions approximately midway between the bottom edge and the top edge of the side wall. In some embodiments, a canister for liquid cryogen storage includes a series of apertures in a circular side wall, wherein the series of apertures are positioned around the circular side wall at positions approximately one third of the distance from the bottom edge relative to the top edge of the side wall. In some embodiments, a canister for liquid cryogen storage includes a series of apertures in a circular side wall, wherein the series of apertures are positioned around the circular side wall at positions between approximately midway between the bottom edge and the top edge of the side wall and approximately one third of the distance from the bottom edge relative to the top edge of the side wall.

In some embodiments, a canister for liquid cryogen storage includes a flange affixed to an interior surface of the circular side wall at a position adjacent to the apertures, and wherein the flange affixed to an interior surface of the circular side wall is positioned to substantially cover the series of apertures in the circular side wall. In some embodiments, a canister for liquid cryogen storage includes a flange affixed to an interior surface of the circular side wall at a position adjacent to the apertures, and wherein the flange affixed to an interior surface of the circular side wall is positioned to partially cover the series of apertures in the circular side wall. In some embodiments, a canister for liquid cryogen storage includes a flange affixed to an interior surface of the circular side wall at a position adjacent to the apertures, and wherein the flange affixed to an interior surface of the circular side wall is affixed to the interior surface of the circular side wall at a position above the series of apertures.

In some embodiments, a canister for liquid cryogen storage further includes a permeable membrane positioned within the bottom wall. The permeable membrane can include, for example, a sintered metal. The permeable membrane can include, for example, a membrane permeable to liquid nitrogen. The permeable membrane can, for example, be permeable to liquid nitrogen at a rate sufficient to allow the cylindrical cup to fill in an hour when the cylindrical cup is submerged in liquid nitrogen. The permeable membrane can, for example, be permeable to liquid nitrogen at a rate sufficient to allow the cylindrical cup to fill in a period longer than a minute when the cylindrical cup is submerged in liquid nitrogen. In some embodiments, a canister for liquid cryogen storage further includes a handle affixed to the canister.

In some embodiments, a canister for liquid cryogen storage includes: a cylindrical cup with a circular side wall and a bottom wall; and a layer of absorbent material affixed to the interior surface of the circular side wall, the absorbent material absorbent to a liquid cryogen. In some embodiments, the cylindrical cup includes: a cylindrical cup of a size and shape to fit through the neck of a Dewar and to be retained within the inner storage region of the Dewar. In some embodiments, the cylindrical cup includes: a cylindrical cup of a size and shape to retain a plurality of bovine semen straws.

In some embodiments, a canister for liquid cryogen storage includes a layer of absorbent material affixed to an interior surface of a circular side wall, the absorbent material absorbent to a liquid cryogen. The absorbent material can, for example, include a material that absorbs liquid nitrogen. The absorbent material can, for example, include a

felted material. The absorbent material can, for example, include a fiberglass mesh material. The absorbent material can, for example, include an aerogel material. The absorbent material can, for example, include a cryogen-permeable material with porosity.

In some embodiments, a canister for liquid cryogen storage further includes a cryogen-permeable region within the bottom wall. The cryogen-permeable region within the bottom wall can include, for example, a group of apertures within the bottom wall. The cryogen-permeable region within the bottom wall can include, for example, a sintered metal. The cryogen-permeable region within the bottom wall can include, for example, a group of apertures within the bottom wall.

In some embodiments, a canister for liquid cryogen storage further includes a permeable layer positioned adjacent to the layer of absorbent material at a surface of the layer of absorbent material opposite to the canister. The permeable layer can include, for example, a layer of metal including a plurality of apertures. In some embodiments, a canister for liquid cryogen storage further includes a layer of absorbent material affixed to the interior surface of the bottom wall. In some embodiments, a canister for liquid cryogen storage further includes a handle affixed to the canister.

In some embodiments, a canister for liquid cryogen storage includes: a cylindrical cup with a circular side wall and a bottom wall; and a solid mass of a size and shape to substantially fill the interior of the cylindrical cup, the solid mass including a plurality of cavities positioned vertically in the solid mass. In some embodiments, the cylindrical cup includes: a cylindrical cup of a size and shape to fit through the neck of a Dewar and to be retained within the inner storage region of the Dewar. In some embodiments, the cylindrical cup includes: a cylindrical cup of a size and shape to retain a plurality of bovine semen straws.

In some embodiments, a canister for liquid cryogen storage includes a cylindrical cup with a circular side wall and a bottom wall, and wherein the cylindrical cup includes a cryogen-permeable region within the bottom wall. In some embodiments, the cryogen-permeable region within the bottom wall includes a group of apertures in the bottom wall.

In some embodiments, a canister for liquid cryogen storage includes a solid mass of a size and shape to substantially fill the interior of the cylindrical cup, the solid mass including a plurality of cavities positioned vertically in the solid mass. In some embodiments, the solid mass is a thermal mass. In some embodiments, the solid mass is fabricated with metal. The plurality of cavities positioned vertically in the solid mass can

include, for example, a plurality of cavities of a size and shape to contain one or more semen straws. The plurality of cavities can be, for example, positioned so that a user of the container can quickly identify and remove particular semen straws. In some embodiments, a canister for liquid cryogen storage further includes a handle affixed to the canister.

Results- Protection of Semen from Damage

Since Prototype 2, based on Design 2, demonstrated improvement in maintaining temperature and reducing the loss of LN during testing, we tested its ability to protect against semen damage in comparison to the standard canister. Both a standard canister and Prototype 2, based on Design 2, were subjected to multiple ambient temperature exposures similar to those described herein. Semen stored in the Prototype 2 canister showed less damage by post-thaw acrosome integrity measurements (*see* Figures 12A & 12B). These data strongly suggest that a canister lined with an LN absorbent material can better maintain the fertility of frozen semen when subjected to repeated poor handling.

Herein we show that damage associated with improper access of cryogenically stored bovine semen is mitigated with simple and inexpensive improvements to the storage system. An alternative solution to this problem is to train AI technicians and semen handlers to avoid exposing frozen semen straws to non-LN temperatures for more than a few seconds. While proper technique is stressed in many training programs, the problem of exposure-induced damage persists and is suspected to be more severe in the developing world.

It is likely that the majority of semen damage caused during improper frozen semen access is inadvertent. Inadequately trained users might assume that exposing frozen semen to ambient conditions is not damaging as long as the contents remains in a frozen state. However, thermal damage can occur after very brief ambient exposures that are not sufficient to thaw the semen.

Studies suggest that damage due to brief ambient exposures results when the bulk product temperature rises above -137^0 C (Debenedetti, P. G. Supercooled and glassy water, *J. Phys.-Condes. Matter* **15,** R1669-R1726 (2003), which is incorporated herein by reference) —the glass transition temperature of water—and then cools back down when re-introduced to LN (Stroud, B., Consequences of mishandling frozen semen and embryos in *Proceedings, Applied Reproductive Strategies in Beef Cattle – Northwest* 191-204

(2012) and Berndtson, W. E., Pickett, B. W., Rugg, C. D. & Collins, F. Procedures for Field Handling of Bovine Semen in Plastic Straws, 51-60 (1976), which are each incorporated by reference). The reordering of water molecules during these events likely affects multiple aspects of semen biology associated with fertilizing potential. While these insults are presumably stochastics, they appear to have a greater chance of negatively affecting the integrity of biological membranes since we observed damage via the acrosomal assay but not the motility or viability assays. The exact mechanism of this phenomenon has yet to be fully elucidated, but it may share similarities to cryocapacitation, a form of cryoinjury that can occur during the freezing process that largely affects sperm outer and acrosomal membranes (Bailey, J., Bilodeau, J. & Cormier, N., Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon minireview, *J. Androl.* **21,** (2000) and Partyka, A., Niżański, W. & Ochota, M. in *Current Frontiers in Cryobiology* (ed. Katkov, I. I.) 547-575 (InTech, 2012). doi:10.5772/1962, which are each incorporated by reference).

In this study we exposed frozen bovine semen to poor practices that mimic improper access, and subsequently measured semen damage. The relationship between exposure time and acrosome damage is complex but our data are consistent with the hypothesis that damage accumulates upon fluctuations about the glass and freezing transitions. To decrease thermal fluctuations within the canister contents, we designed several simple and inexpensive improvements to the Dewar canister. These improvements, such as canisters lined with an LN absorbent material, better maintain characteristics of semen that are associated with fertilizing potential.

We hesitate to extrapolate the effect that this technological mitigation would have on actual fertilization rates. Poor handling is one of several factors that affect AI success and poor handling behaviors occur with variable frequencies and severities, making it difficult to quantify their full effect. Furthermore, we constrained our evaluation to a select number of aspects of sperm biology that are necessary for fertilization (Ikawa, M., Inoue, N., Benham, A. M. & Okabe, M. Fertilization: A sperm's journey to and interaction with the oocyte, *J. Clin. Invest.* **120,** 984-994 (2010), which is incorporated herein by reference). Of the biological aspects we investigated, we suspect that the assays do not fully reflect damage that poor handling imparts. For example, the results from the acrosomal integrity assay are reliant on gross morphological differences in these

structures. We suspect that this assay underestimates the level of acrosomal damage by failing to register submicroscopic injuries that negatively affect fertility.

Since all current *in vitro* semen assays suffer from some degree of sensitivity limitations and because each assay measures only a subset of aspects needed for a semen straw to result in heifer fertilization—including in vitro fertilization (Ball, G. D. *et al.* Factors affecting successful in vitro fertilization of bovine follicular oocytes, *Biol. Reprod.* **28,** 717–725 (1983), which is incorporated by reference herein) — predicting the fertilizing potential of a semen straw in a laboratory setting is a dubious exercise (Rodrıguez,-Martinez, H. Can We Increase the Estimative Value of Semen Assessment. *Reproduction Domest. Anim.* **41,** 2-10 (2006); Oliveira, L. Z., Monteiro, F. M., Carla, E. & Celeghini, C. *Success in Artificial Insemination - Quality of Semen and Diagnostics Employed. inntech open* (2013) and Rodriguez-Martinez, H., Laboratory Semen Assessment and Prediction of Fertility: still Utopia?, *Reprod. Domest. Anim.* **38,** 312-318 (2003), which are each incorporated by reference herein). While we are reluctant to quantify the impact of our Dewar improvements without a full-fledged field study, our results imply it will lead to higher rates of AI success.

The intention of this study was to find technological solutions that would lead to a higher AI success rate—and therefore higher farmer incomes—in the developing world. Of course, any technological solution will need to pass several hurdles, in addition to such a technical evaluation, for it to result in this goal. Among these challenges are those associated with any requirements on the user to interact with the equipment differently than they currently do.

In a field observation we sought feedback regarding prototypes based on all three designs. Our assessment of the improved canisters was carried out in Kenya where we conducted 17 one-on-one interviews, mock insemination workstations with AI technicians, and 30 interviews in group settings that included seventeen practicing AI technicians, four semen distributers, two senior AI educators and seven leaders, managers and policy directors in the dairy sector in Kenya. All observations and interviews found no observable difference in how the prototype canisters were handled or managed as compared to standard equipment (data not shown).

Among the learnings from this evaluation was the observation that AI technicians in rural Kenya invariably operate with partially filled LN Dewars. To ensure that the performance of the prototypes mentioned herein do not suffer as a result of this behavior

(intended to save on LN costs), we repeated thermal performance and acrosomal integrity assays with partially filled Dewars. Results from these studies suggest that the protection that the Prototype 2 canister imparts is minimally affected by the amount of LN in the Dewar (*see* Figures 11D and 12B).

Our findings are applicable to industries outside of the AI industry as well. Maintenance of the cold chain is crucial to preserve the integrity of a variety of biological samples such as seeds, oocytes, blood products, embryos, stem cells, and tissues from humans, animals and plants (Hubel, A., Spindler, R. & Skubitz, A. P. N., Storage of human biospecimens: selection of the optimal storage temperature. *Biopreserv. Biobank.* **12,** 165-75 (2014), which is incorporated herein by reference). Inventory control methods such as those described here could limit thermal fluctuations within these samples and protect them from cryodamage. These technologies may be especially useful for samples frozen using vitrification, a process often used in the preservation of oocytes, embryos, and stem cells, which leaves the samples especially sensitive to devitrification damage (Sansinena, M., Santos, M. V., Taminelli, G. & Zaritky, N., Implications of storage and handling conditions on glass transition and potential devitrification of oocytes and embryos, *Theriogenology* **82,** 373-378 (2014), which is incorporated herein by reference).

Examples

Reagents

Unless otherwise indicated, reagents used in this study were purchased from Sigma (St. Louise, MO). All frozen semen used in the study was purchased from Accelerated Genetics (Baraboo, WI). Unless otherwise indicated, semen within an experiment utilized straws from a single lot, which in some tests were from the Holstein Bull Michigan Frost (014HO07313) and packaged in 0.5 ml French straws.

Example 1: Quantifying Semen Damage

Figure 5 depicts post-thaw characteristics of frozen semen subjected to repeated ambient temperature exposures. Frozen straws from the same lot of semen were stored in the canister of a standard 3 L Dewar and subjected to repeated one minute ambient temperature exposures. Measurements of membrane viability staining, sperm motility, and acrosome integrity are displayed in Figure 5. Membrane viability and sperm motility data points were measured on three samples. Error bars represent standard deviation (SD).

Each acrosome integrity data point was measured on samples from a single straw. Error bars are not included on the acrosome integrity data set.

To quantify the damage associated with these types of repeated exposures, we subjected the goblet of a standard 3 L Dewar containing frozen semen to recurring ambient temperature exposures (23° C ± 1° C) and repeated these treatments at various exposure durations. Between each exposure, the canister was submerged in a LN-filled Dewar for at least one minute. After these cycling treatments were complete, we measured aspects of semen biology that correlate with fertility (H. Rodriguez,-Martinez, "Can We Increase the Estimative Value of Semen Assessment," Reproduction Domest. Anim., Vol. 41, pp. 2-10, 2006 and L. Z. Oliveira, F. M. Monteiro, E. Carla, and C. Celeghini, Success in Artificial Insemination - Quality of Semen and Diagnostics Employed. 2013, which are each incorporated by reference).

Both semen motility and outer membrane viability staining were resistant to these treatments (see Figure 6). A relatively large number of one minute exposures were required to induce measureable damage in these physiological characteristics.

However, damage to the acrosome was much more pronounced (see Figures 5 and 6). The acrosome is a large vesicle located at the anterior region of the sperm head that contains hydrolytic enzymes and surface antigens necessary for the acrosome reaction, a necessary process in fertilization, where the sperm penetrates the zona pellucida of the egg (M. Ikawa, N. Inoue, A. M. Benham, and M. Okabe, "Fertilization: A sperm's journey to and interaction with the oocyte," J. Clin. Invest., Vol. 120, No. 4, pp. 984-994, 2010 which is incorporated by reference).

Measurements showed direct relationships between acrosome damage and exposure cycle number and duration (see Figure 6). These data are consistent with reports that show that these structures are particularly sensitive and dynamic (J. Bailey, J. Bilodeau, and N. Cormier, "Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon minireview," J. Androl., Vol. 21, No. 1, 2000 which is incorporated by reference).

Example 2: Quantifying Semen Damage

Generally, Dewar canisters contain multiple semen straws. If an AI technician uses poor handling practices then each time he or she removes a straw for use, the remaining straws within the inventory will experience multiple thermal exposures (*see, e.g.* Figure

2). Similar events could occur during the transfer of straws between Dewars, such as when importers transfer straws to distribution centers, distributers transfer straws to AI technicians, etc.

To quantify the damage associated with repeated thermal exposures, we subjected the canister of a standard 3 L Dewar containing frozen bovine semen to recurrent ambient temperature exposures. We repeated these cycling treatments at various exposure durations, and between each exposure the canister was submerged in LN. After cycling treatments were complete, we measured aspects of semen biology that correlate with fertility (Rodrıguez,-Martinez, H. Can We Increase the Estimative Value of Semen Assessment, *Reproduction Domest. Anim.* **41,** 2-10 (2006) and Oliveira, L. Z., Monteiro, F. M., Carla, E. & Celeghini, C., *Success in Artificial Insemination - Quality of Semen and Diagnostics Employed. inntech open* (2013), which are each incorporated by reference). Both semen motility and viability measurements were resistant to these treatments (*see* Figure 2A). Surprisingly, 20 one minute ambient exposures were not sufficient to induce measureable damage in these physiological characteristics.

However, damage to the acrosome was much more pronounced (see Figure 7). The acrosome is a large vesicle located at the anterior region of the sperm head that contains hydrolytic enzymes and surface antigens necessary for the acrosome reaction, a necessary process in fertilization where the sperm penetrates the zona pellucida of the egg (Ikawa, M., Inoue, N., Benham, A. M. & Okabe, M. Fertilization: A sperm's journey to and interaction with the oocyte, *J. Clin. Invest.* **120,** 984-994 (2010), which is incorporated herein by reference). Measurements showed that acrosome damage increased with the quantity and duration of thermal exposure (see Figure 8). These data are consistent with reports that show that acrosomes are dynamic structures particularly sensitive to damage (Bailey, J., Bilodeau, J. & Cormier, N., Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon minireview, *J. Androl.* **21,** (2000) and Stroud, B. Consequences of Handling Frozen Semen and Embryos. in *Proceedings, Applied reproductive strategies in Beef Cattle,* 191-204 (2012), which are each incorporated herein by reference).

Figures 7 and 8 show that poor handling damages semen. For the data shown in Figure 7, frozen semen straws were subjected to repeated one minute ambient temperature exposures. Measurements of membrane viability staining, sperm motility, and acrosome integrity are displayed. For the data shown in Figure 8, frozen semen was subjected to

multiple ambient temperature exposures at 0.5, 1, 2, and 4 minute durations. The post-thaw acrosome integrity measurements are shown. For both graphs, the fitted lines are included as visual guides (n = 6, error bars represent standard deviation (SD)).

Example 3: Motility and Membrane Viability Staining

Frozen semen straws were thawed in a water bath set at 35° C for 30 seconds. Motility and membrane viability staining was performed using computer assisted semen analysis (CASA). Briefly, for motility staining, 20 µL of semen was gently mixed with 30 µL Easybuffer B (IMV Technologies, Maple Grove MN) and 50 µL of 1 mg/ml Hoechst 33342. Samples were then incubated at 35° C for 20 minutes, loaded onto prewarmed four chamber Leja slides (IMV Technologies) and imaged using the Animal Motility package on an IVOS II (Hamilton Thorne, Beverly, MA) system using version 1.5 of the CASA II sperm analysis software and the manufacturer's recommended settings.

For membrane viability staining, semen was thawed as previously described. 20 µL of semen was gently mixed with 30 µL Easybuffer B (IMV Technologies) and 50 µL of 1 mg/ml Hoechst 33258. Samples were then incubated at 35° C for 2 minutes, loaded onto prewarmed four chamber Leja slides and imaged using the Animal Motility Viadent package on the same IVOS II system and software.

Example 4: Acrosome Integrity

Acrosome integrity measurements were adapted from previously described methods (see: E. C. C. Celeghini, R. P. De Arruda, a. F. C. De Andrade, J. Nascimento, and C. F. Raphael, "Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes," Reprod. Domest. Anim., Vol. 42, No. 5, pp. 479-488, 2007; and J. K. Graham, "Assessment of sperm quality: A flow cytometric approach," Anim. Reprod. Sci., Vol. 68, No. 3-4, pp. 239-247, 2001, which are each incorporated herein by reference.) Frozen semen straws were thawed in a water bath set at 35° C for 30 seconds. Semen was then transferred to 1.7 mL microcentrifuge tube and incubated at 35° C for one hour.

In some instances, samples were then cooled to 4° C and centrifuged 500 RCF for 5 minutes at 4° C. Supernatants were then discarded and the pellet was gently resuspended in 1 mL PBS supplemented with 10% bovine serum albumin (BSA) and 25 µg/mL fluorescein conjugated peanut agglutinin (PNA-FITC). Samples were incubated for

30 minutes at 4° C. Samples were washed with PBS supplemented with 10% BSA, resuspended in 0.5 mL Cytofix (BD Biosciences, Franklin Lakes, NJ), and incubated for 15 minutes at 4° C. Samples were then centrifuged 500 RCF for 5 minutes at 4° C, and the pellets were resuspended in PBS supplemented with 10% BSA. 20 µL of this suspension was spread on a microscope slide and mixed with a drop of Fluoroshield with DAPI mounting medium. Acrosome integrity was measured using fluorescence microscopy as previously described (E. C. C. Celeghini, R. P. De Arruda, a. F. C. De Andrade, J. Nascimento, and C. F. Raphael, "Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes," Reprod. Domest. Anim., Vol. 42, No. 5, pp. 479-488, 2007, which is incorporated by reference).

In some instances, after the semen was transferred to 1.7 mL microcentrifuge tube and incubated at 35° C for one hour, 20 µL of semen solution was spread on a microscope slides and air-dried for 15 min. The slides were fixed by immersion in absolute methanol for 15 min. Slides were rinsed in PBS baths twice for 5 min each, transferred to a bath containing 25 µg/mL PNA-FITC for 30 min, and rinsed in three PBS baths for five minutes each. Slides were gently dried using compressed air. One drop of Fluoroshield™ with DAPI Histology Mounting Medium was then applied to each slide and acrosome integrity was measured using fluorescence microscopy as previously described (Celeghini, E. C. C., De Arruda, R. P., De Andrade, a. F. C., Nascimento, J. & Raphael, C. F. Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes, *Reprod. Domest. Anim.* **42,** 479-488 (2007); P. Lybaert, A. Danguy, F. Leleux et al., Improved methodology for the detection and quantification of the acrosome reaction in mouse spermatozoa, Histol Histopathol (2009) 24: 999-1007; S. Esteves, R. Sharma, A. Thomas, A. Agarwal. Evaluation of Acrosomal Status and Sperm Viability in Fresh and Cyropreserved Specimens by the Use of Fluorescent Peanut Agglutinin Lectin in conjunction with Hyo-osmotic Swelling Test. International Braz J Urol, 33 (3): 364-376, 2007; and Carrel, Aston Eds. Spermatogenesis: Methods and Protocols, Methods in Molecular Biology, Vol. 927 (2013) which are each incorporated herein by reference).

Example 5: Instrumented Semen Straw

A bundle of three 36 AWG Type T thermocouples was inserted into a semen straw. The thermocouple tips were positioned 34 mm, 79 mm, and 124 mm from the crimped end of the straw. The bundle was secured to the crimped end of the straw using 19 mm Kapton tape, waxed lacing cord, and tapered round rubber plugs.

Each thermocouple was connected to a National Instruments data acquisition system with a Labview interface and sampled at 1 Hz. All thermocouples were two-point-calibrated using a water bath and LN bath.

Example 6: Prototypes

Prototypes based of all three designs were constructed and subjected to ambient exposure tests described below. In this study the holes on the Design 1 prototype were located at the canister midpoint. A vapor guard and permeable membrane are not included in this data set. The Design 2 prototype used a standard canister fitted snuggly with a 9.5 mm thick cabosil infused blanket against the base and walls. LN was able to drain out when raised from the Dewar. The Design 3 prototype fitted an aluminum block with twenty 3.2 mm thru holes into a standard canister.

Standard canisters of a YDS-3 Dewar (Chart, Garfield Heights, OH) were used as the platform for prototype construction. The canister for Prototype 1 was modified by welding a circular piece of sheet metal to seal the canister bottom and drilling 3.175 mm diameter holes at the canister midpoint. A custom aluminum ring, 2.1 mm thick, was designed to fit snuggly on the inside of the canister, 5 mm above the holes at the midpoint extending 12 mm downward (*see* Figure 3B and 4B). A 1.25 mm gap allowed the LN to flow through the holes at the midpoint.

Prototype 2 was fitted snuggly with a 5 mm thick Cryogel® (Aspen Aerogels, Northborough, MA) blanket against the base and walls.

Prototype 3 fitted an aluminum block with eight 7.2 mm thru holes into a standard canister.

Each test was carried out by placing three straws instrumented with thermocouples in a canister. Each instrumented straw had three 36 gauge type T thermocouples that were positioned at the bottom, middle, and top of the straw. Each canister was raised by hand out of the Dewar into the environment and held for approximately one minute then re-submerged into the Dewar. Figure 10 compares the thermal response of each design with the standard canister as a baseline. All three designs demonstrate an improvement

delaying a temperature rise in the straws. Of note all three prototypes are very effective at keeping the mid and lower portion of the straw near the LN temperature. Designs 2 and 3 appear to be slightly more effective at delaying the temperature rise in the top portion of the straw. Additional experiments are planned to confirm this finding. The subject experiment was performed in a laboratory held at 23° C ± 0.2° C throughout the experiment. The effect of convection from small oscillations while holding the canister and cross flow from the air handling system were not measured. However, thermal response simulations using Comsol, a multiphysics modeling and simulation tool, suggest that natural convection is a driving factor and likely marginalizes potential forced convection effects in our laboratory experiment. Further testing is planned to quantify the effect of wind and direct solar radiation on the prototypes' thermal response.

Testing to compare LN loss by cycling the canisters was also carried out by measuring the change in mass of the entire system, *i.e.* the Dewar and canister (see Figure 9). As a reference the standard canister loses approximately 16 g of LN which is similar to the Design 1 prototype. The Design 2 prototype loses approximately 13 g, a slight improvement on the standard canister while Design 3 consumes slightly more LN. Mass loss is averaged over three trials with all data within ±2 g of the respective mean. We note that the LN mass loss for a given design is a combination of LN that evaporates when the canister is exposed to the environment and from the boiling that occurs when the canister is re-inserted into the Dewar.

Figure 9 is a comparison of LN loss after poor handling. Canisters were removed from a full 3 L Dewar for one minute then reintroduced to the system. The loss in mass is shown (n=3, error bars represent SD). Figure 10 shows a graph of temperature as a function of time for straws in the Design 1, 2, and 3 prototypes removed from LN in a 3 L Dewar to ambient temperature for approximately 1 min after which time, canisters are re-submerged into LN. The standard canister is shown for reference.

Example 7: Thermal Performance

We subjected the prototypes to ambient exposure tests by placing thermocouple temperature sensors at the top, middle, and bottom of a semen straw. Each prototype contained three straws with thermocouples. We raised the canister prototypes completely out of a LN-filled Dewar for approximately one minute then re-submerged into the Dewar.

The experiment was performed in a laboratory held at 23^0 C ± 0.2^0 C throughout the experiment.

Figure 11 shows that prototypes reduce thermal fluctuations within semen straws during poor handling. Temperature measurements at the top (T), middle (M), and bottom (B) of a semen straw placed within Prototype (P) 1 representing Design 1, Prototype 2 representing Design 2 and Prototype 3 representing Design 3 (subfigures A, B, and C, respectively) removed from a full 3 L Dewar for approximately one minute then re-submerged into LN are shown. Readings from similar experiments using a standard canister (SC) are shown for reference. D. The experiment in subfigure B was repeated with a Dewar that was 25% full of LN. All plots report values as mean readings from thermocouples located on three different semen straws from the same ambient exposure. Data are representative of three independent experiments.

All three prototypes demonstrated an improvement in delaying a temperature rise in the straws (*see* Figure 11). The three prototypes effectively maintained the mid and lower portion of the straw near the -196^0 C LN temperature. Prototypes 2 and 3 were slightly more effective at retaining the temperature in the top portion of the straw. The effect of convection from small oscillations while holding the canister and cross flow from the air handling system were not measured. However, thermal response simulations using a multiphysics modeling and simulation tool (COMSOL, Stockholm, Sweden) suggest that natural convection is a driving factor and likely marginalizes potential forced convection effects in this experiment (data not shown).

Figure 11 is a set of graphs of testing results indicating that prototypes reduce thermal fluctuations within semen straws during poor handling. Temperature measurements at the top (T), middle (M), and bottom (B) of a semen straw placed within Design (P) 1, 2 and 3 (subfigures A, B, and C, respectively) removed from a full 3 L Dewar for approximately one minute then re-submerged into LN are shown. Readings from similar experiments using a standard canister (SC) are shown for reference.

Figure 11D. The experiment in subfigure 11B was repeated with a Dewar that was 25% full of LN. All plots report values as mean readings from thermocouples located on three different semen straws from the same ambient exposure. Data are representative of three independent experiments.

To compare LN loss due to cycling the canisters, we measured the change in mass of the entire system, *i.e.* the Dewar and canister (*see* Figure 9). The standard canister

results in the loss of approximately 16 g of LN; we observed a similar loss with the Prototype 1. Prototype 2 lost approximately 13 g, a slight improvement on the standard canister, while Prototype 3 consumed more LN at 21.3 g. We note that the LN mass loss for a given prototype was a combination of LN that evaporated when the canister was exposed to the environment and from the boiling that occurred when the canister was re-inserted into the Dewar.

Example 8: Validation

The ability of the Design 1 prototype to protect against semen damage was tested in a side-by-side experiment with a standard canister. A Design 1 prototype, with holes located at the midline without the vapor guard and permeable membrane, and a standard canister both containing frozen semen from three different bulls were subjected to multiple ambient temperature exposures similar to those previously described. Post-thaw acrosome integrity analysis showed greater damage in the semen from all three bulls when the standard canister was used. These data suggest that an improved canister can better maintain the fertility of frozen semen when subjected to poor handling (see Figure 13).

Figure 9 illustrates mass of liquid nitrogen loss after a canister was removed from a 3 L Dewar for one minute then reintroduced to the system.

At the time of this of submission, experiments to further compare the effects of repeated thermal exposures on frozen semen stored in the prototype canisters were in progress. We intend to include these results in a future presentation.

Figure 13 shows post-thaw acrosome integrity measurements of frozen semen stored in the Design 1 prototype and a standard canister subjected to up to twenty 1 min ambient temperature exposures.

Example 9: Thermal Cycling Experiments

Thermal cycling experiments were performed in a laboratory with ambient conditions measuring 22 ± 1^0 C. A total of six 0.5 ml semen straws occupied the canister for all exposures. For experiments utilizing a full LN Dewar, canisters were returned to the Dewar for at least one minute between exposures. For experiments utilizing a 25% LN Dewar, canisters were returned to the Dewar for at least ten minutes between exposures. Calibration experiments determined that these durations were sufficient to return the semen straw to LN temperatures.

Example 10: Protection of Semen from Damage

Figures 12A & 12B. Prototype 2 protects semen from poor-handling-induced damage. Frozen semen straws were placed within either a standard canister or Prototype 2 in a full (A) or 25% full (B) Dewar and exposed to up to 40 one minute ambient temperature exposures. The post thaw acrosome integrity measurements are shown. Fitted lines are included as visual guides.

All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in any Application Data Sheet, are incorporated herein by reference, to the extent not inconsistent herewith.

While various aspects and embodiments have been disclosed herein, other aspects and embodiments will be apparent to those skilled in the art. The various aspects and embodiments disclosed herein are for purposes of illustration and are not intended to be limiting, with the true scope and spirit being indicated by the following claims.

CLAIMS

1. A canister for liquid cryogen storage, comprising:
a cylindrical cup with a circular side wall and a bottom wall;
a series of apertures in the circular side wall; and
a flange affixed to an interior surface of the circular side wall at a position adjacent to the apertures.

2. The canister for liquid cryogen storage of claim 1, wherein the cylindrical cup comprises:
a cylindrical cup of a size and shape to fit through the neck of a Dewar and to be retained within the inner storage region of the Dewar.

3. The canister for liquid cryogen storage of claim 1, wherein the cylindrical cup comprises:
a cylindrical cup of a size and shape to retain a plurality of bovine semen straws.

4. The canister for liquid cryogen storage of claim 1, wherein the series of apertures in the circular side wall comprise:
the series of apertures positioned around the circular side wall at positions approximately midway between the bottom edge and the top edge of the side wall.

5. The canister for liquid cryogen storage of claim 1, wherein the series of apertures in the circular side wall comprise:
the series of apertures positioned around the circular side wall at positions approximately one third of the distance from the bottom edge relative to the top edge of the side wall.

6. The canister for liquid cryogen storage of claim 1, wherein the series of apertures in the circular side wall comprise:
the series of apertures positioned around the circular side wall at positions between approximately midway between the bottom edge and the top edge of the side wall and approximately one third of the distance from the bottom edge relative to the top edge of the side wall.

7. The canister for liquid cryogen storage of claim 1, wherein the flange affixed to an interior surface of the circular side wall is positioned to substantially cover the series of apertures in the circular side wall.

8. The canister for liquid cryogen storage of claim 1, wherein the flange affixed to an interior surface of the circular side wall is positioned to partially cover the series of apertures in the circular side wall.

9. The canister for liquid cryogen storage of claim 1, wherein the flange affixed to an interior surface of the circular side wall is affixed to the interior surface of the circular side wall at a position above the series of apertures.

10. The canister for liquid cryogen storage of claim 1, further comprising:
a permeable membrane positioned within the bottom wall.

11. The canister for liquid cryogen storage of claim 10, wherein the permeable membrane comprises:
a sintered metal.

12. The canister for liquid cryogen storage of claim 10, wherein the permeable membrane comprises:
a membrane permeable to liquid nitrogen.

13. The canister for liquid cryogen storage of claim 10, wherein the permeable membrane positioned within the bottom wall is permeable to liquid nitrogen at a rate sufficient to allow the cylindrical cup to fill in an hour when the cylindrical cup is submerged in liquid nitrogen.

14. The canister for liquid cryogen storage of claim 10, wherein the permeable membrane positioned within the bottom wall is permeable to liquid nitrogen at a rate sufficient to allow the cylindrical cup to fill in a period longer than a minute when the cylindrical cup is submerged in liquid nitrogen.

15. The canister for liquid cryogen storage of claim 1, further comprising:
a handle affixed to the canister.

16. A canister for liquid cryogen storage, comprising:

a cylindrical cup with a circular side wall and a bottom wall; and

a layer of absorbent material affixed to the interior surface of the circular side wall, the absorbent material absorbent to a liquid cryogen.

17. The canister for liquid cryogen storage of claim 16, wherein the cylindrical cup comprises:

a cylindrical cup of a size and shape to fit through the neck of a Dewar and to be retained within the inner storage region of the Dewar.

18. The canister for liquid cryogen storage of claim 16, wherein the cylindrical cup comprises:

a cylindrical cup of a size and shape to retain a plurality of bovine semen straws.

19. The canister for liquid cryogen storage of claim 16, wherein the layer of absorbent material comprises:

a material that absorbs liquid nitrogen.

20. The canister for liquid cryogen storage of claim 16, wherein the layer of absorbent material comprises:

a felted material.

21. The canister for liquid cryogen storage of claim 16, wherein the layer of absorbent material comprises:

a fiberglass mesh material.

22. The canister for liquid cryogen storage of claim 16, wherein the layer of absorbent material comprises:

an aerogel material.

23. The canister for liquid cryogen storage of claim 16, wherein the layer of absorbent material comprises:

a cryogen-permeable material with porosity.

24. The canister for liquid cryogen storage of claim 16, further comprising:

a cryogen-permeable region within the bottom wall.

25. The canister for liquid cryogen storage of claim 20, wherein the cryogen-permeable region within the bottom wall includes a sintered metal.

26. The canister for liquid cryogen storage of claim 20, wherein the cryogen-permeable region within the bottom wall includes a group of apertures within the bottom wall.

27. The canister for liquid cryogen storage of claim 16, further comprising:
a permeable layer positioned adjacent to the layer of absorbent material at a surface of the layer of absorbent material opposite to the canister.

28. The canister for liquid cryogen storage of claim 27, wherein the permeable layer comprises:
a layer of metal including a plurality of apertures.

29. The canister for liquid cryogen storage of claim 16, further comprising:
a layer of absorbent material affixed to the interior surface of the bottom wall.

30. The canister for liquid cryogen storage of claim 16, further comprising:
a handle affixed to the canister.

31. A canister for liquid cryogen storage, comprising:
a cylindrical cup with a circular side wall and a bottom wall; and
a solid mass of a size and shape to substantially fill the interior of the cylindrical cup, the solid mass including a plurality of cavities positioned vertically in the solid mass.

32. The canister for liquid cryogen storage of claim 31, wherein the cylindrical cup comprises:
a cylindrical cup of a size and shape to fit through the neck of a Dewar and to be retained within the inner storage region of the Dewar.

33. The canister for liquid cryogen storage of claim 31, wherein the cylindrical cup comprises:
a cylindrical cup of a size and shape to retain a plurality of bovine semen straws.

34. The canister for liquid cryogen storage of claim 31, wherein the cylindrical cup comprises:
a cryogen-permeable region within the bottom wall.

35. The canister for liquid cryogen storage of claim 34, wherein the cryogen-permeable region within the bottom wall comprises:
a group of apertures in the bottom wall.

36. The canister for liquid cryogen storage of claim 34, wherein the cryogen-permeable region within the bottom wall comprises:
a group of apertures in the bottom wall.

37. The canister for liquid cryogen storage of claim 31, wherein the solid mass comprises:
a thermal mass.

38. The canister for liquid cryogen storage of claim 31, wherein the solid mass comprises:
a structure fabricated with metal.

39. The canister for liquid cryogen storage of claim 31, further comprising:
a handle affixed to the canister.

FIG. 1

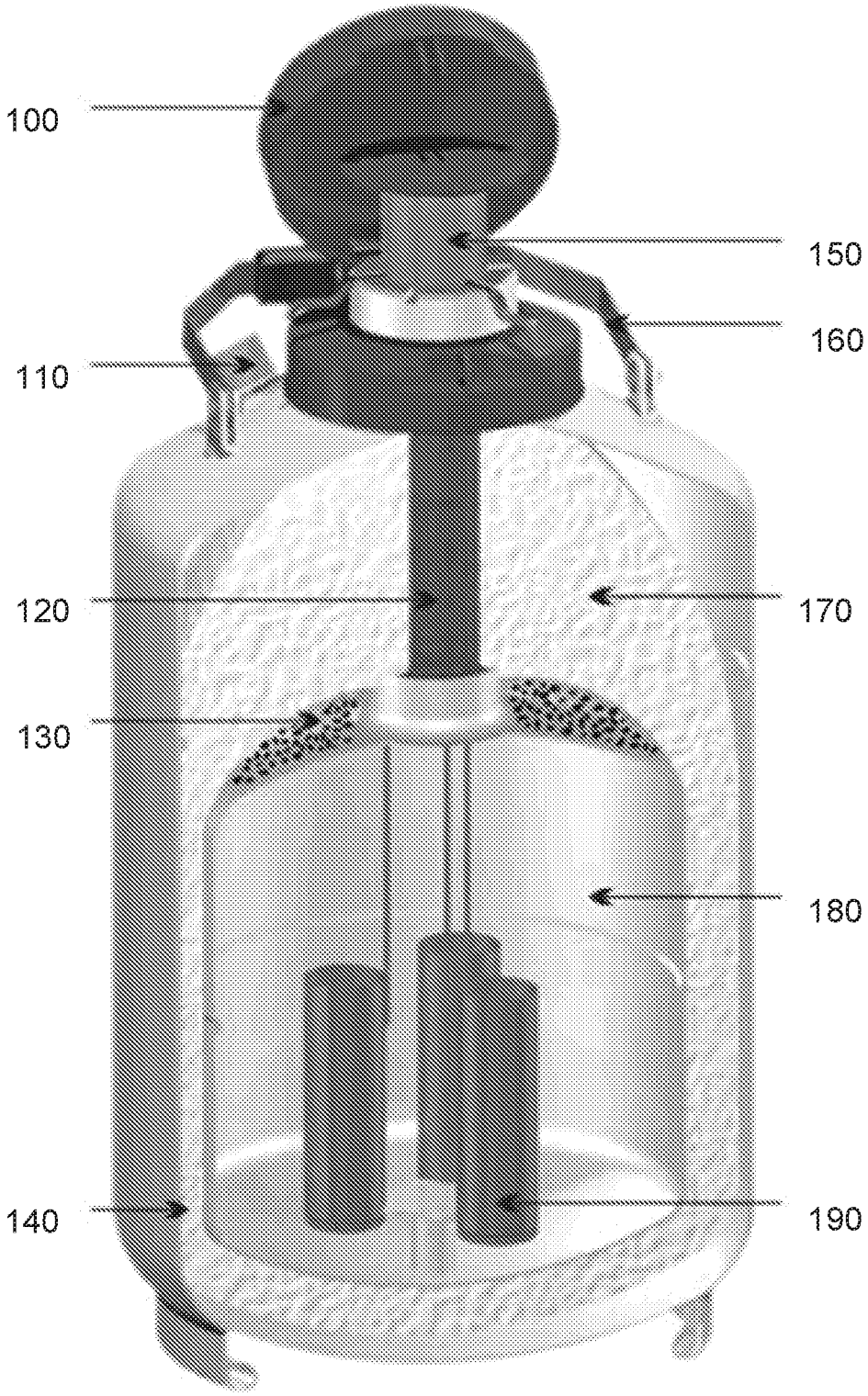


FIG. 2

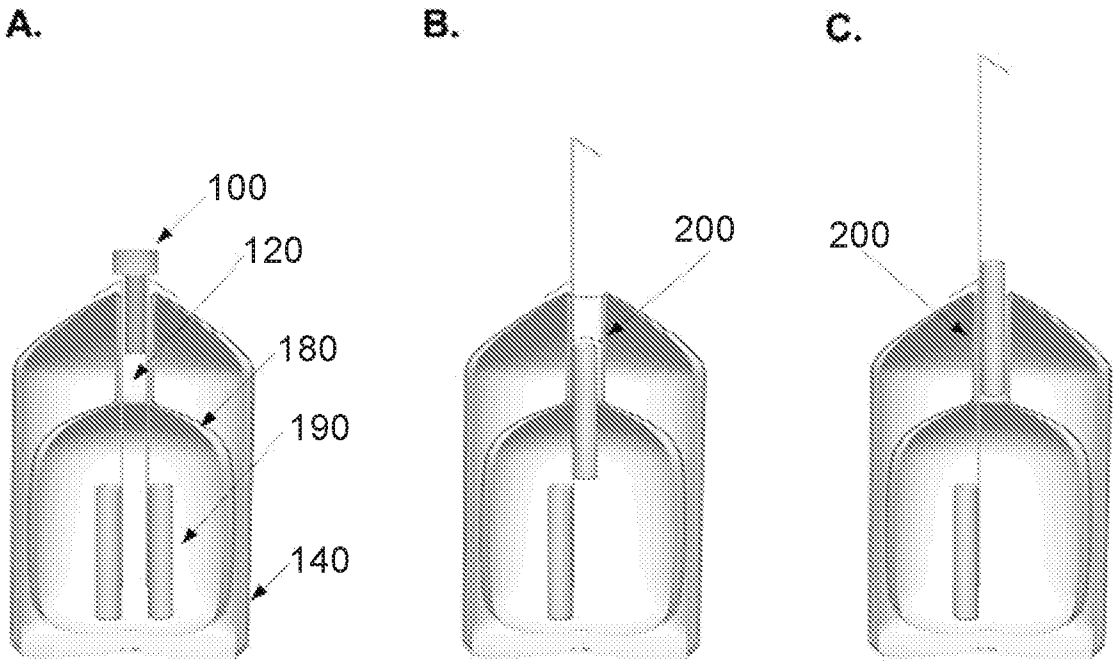


FIG. 3

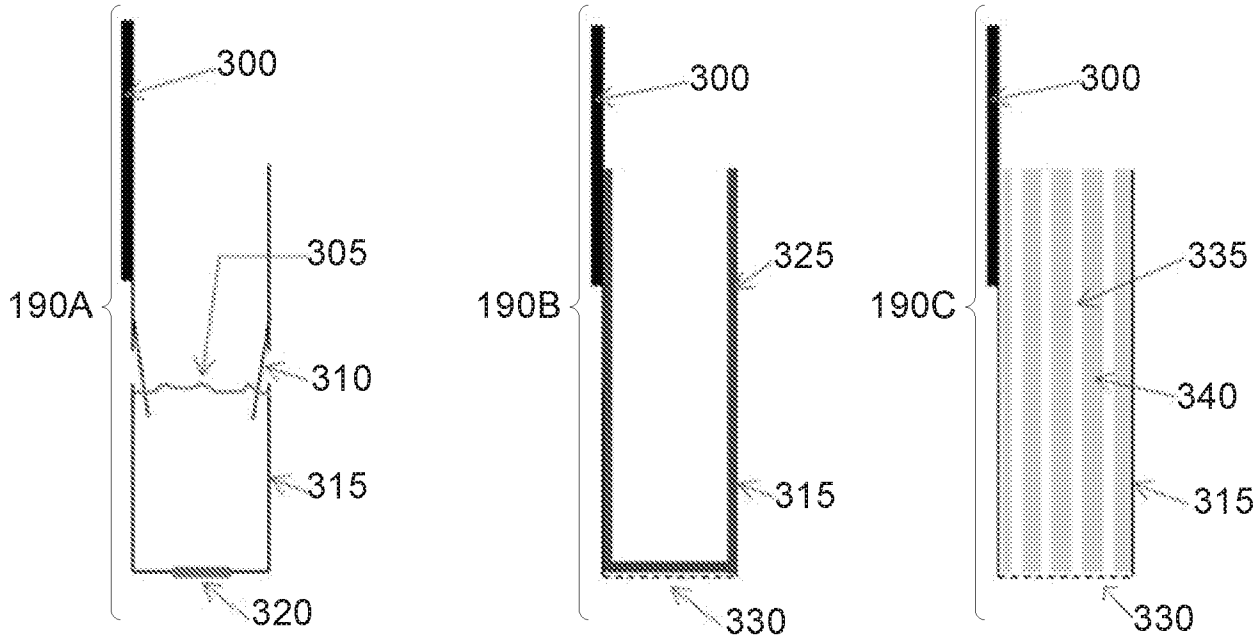

190A
300
305
310
315
320
190B
300
325
315
330
190C
300
335
340
315
330

FIG. 4

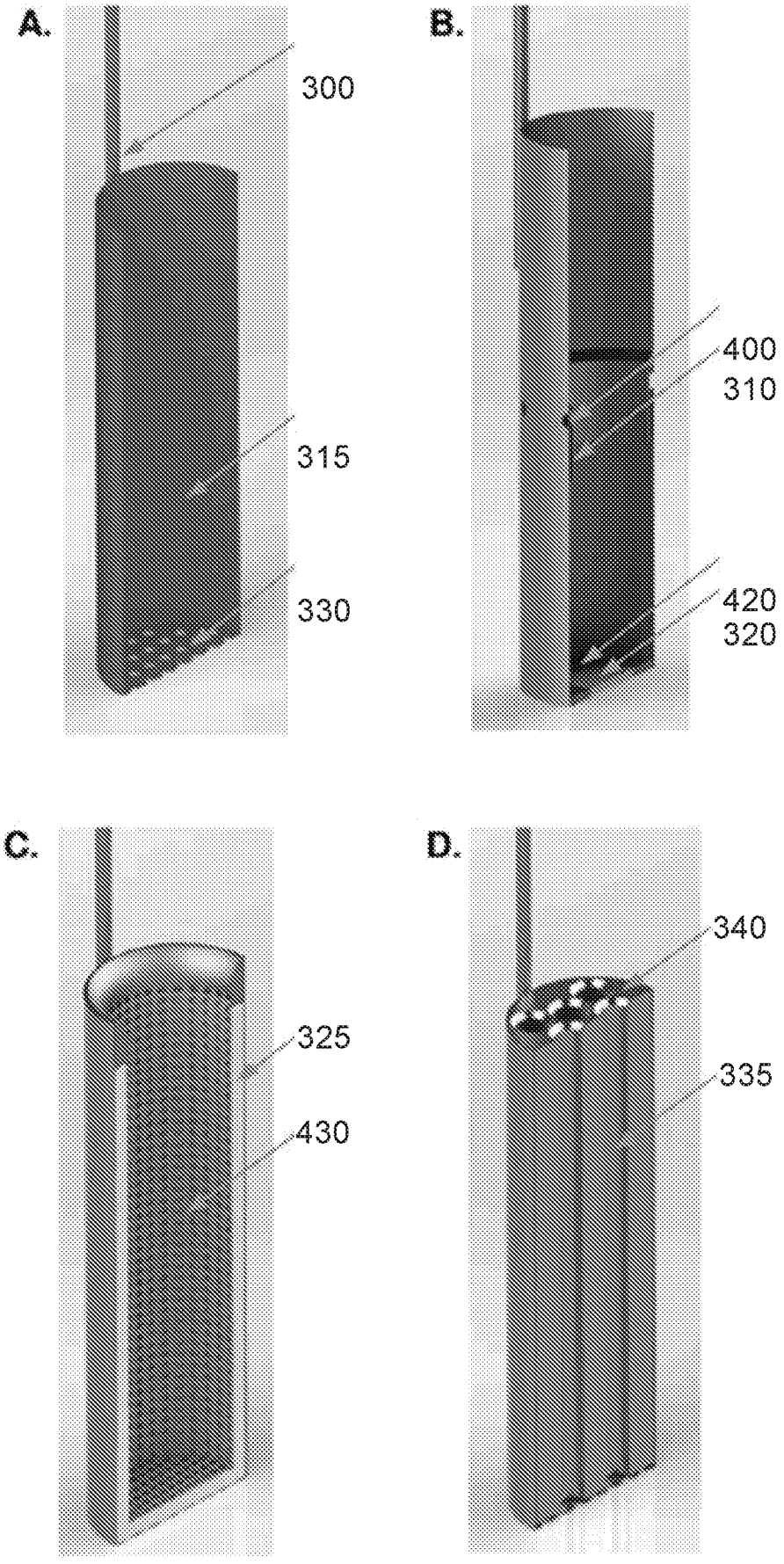


FIG. 5

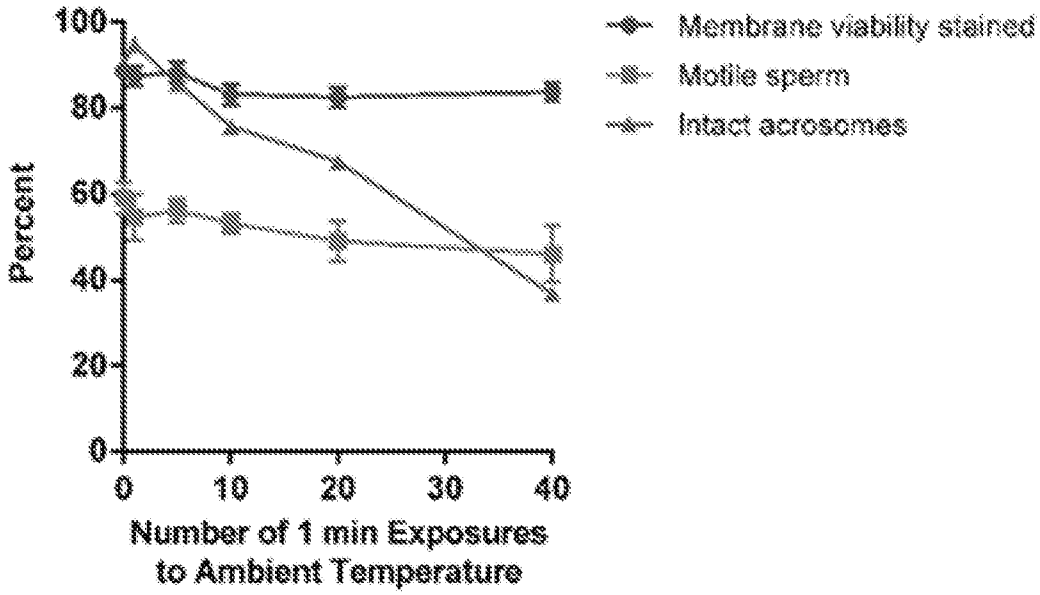

100
80
60
40
20
0
Percent
0
10
20
30
40
Number of 1 min Exposures
to Ambient Temperature
Membrane viability stained
Motile sperm
Intact acrosomes

FIG. 6

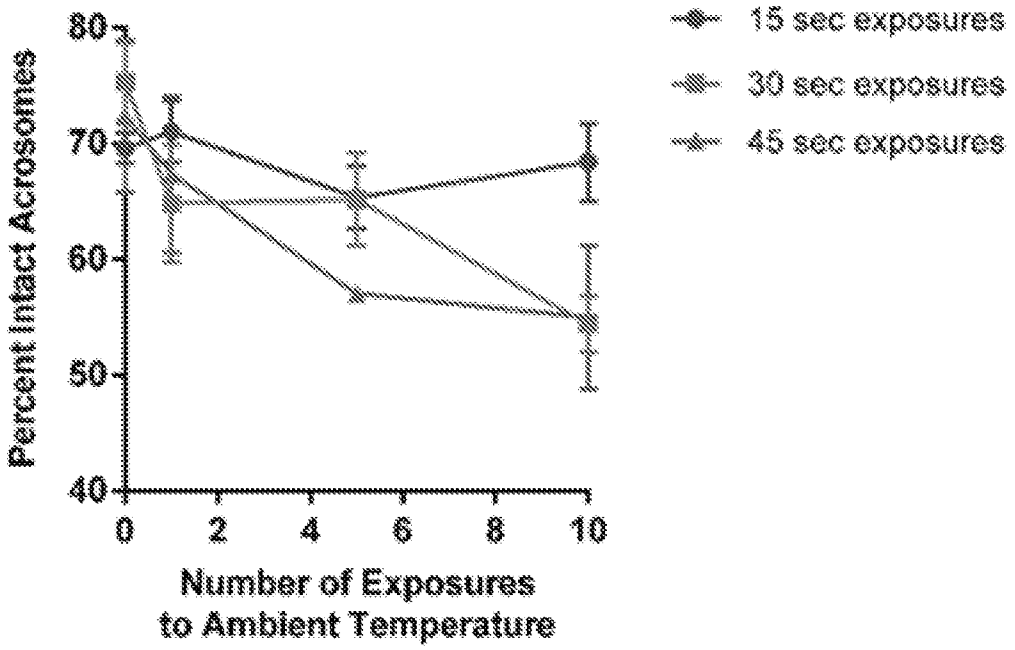

15 sec exposures
30 sec exposures
45 sec exposures
Percent Intact Acrosomes
80
70
60
50
40
0
2
4
6
8
10
Number of Exposures
to Ambient Temperature

FIG. 7

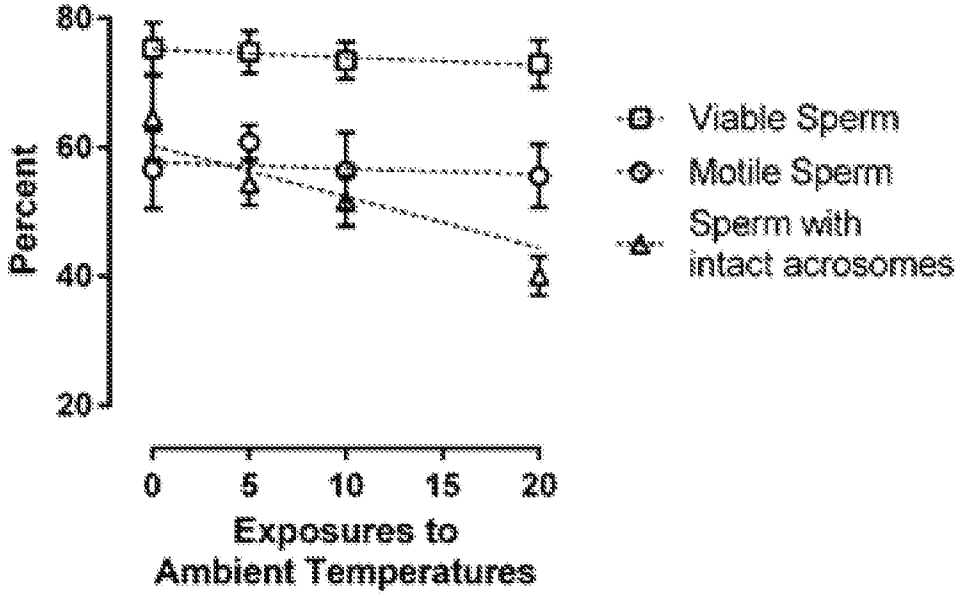

80
60
40
20
Percent
0
5
10
15
20
Exposures to
Ambient Temperatures
Viable Sperm
Motile Sperm
Sperm with
intact acrosomes

FIG. 8

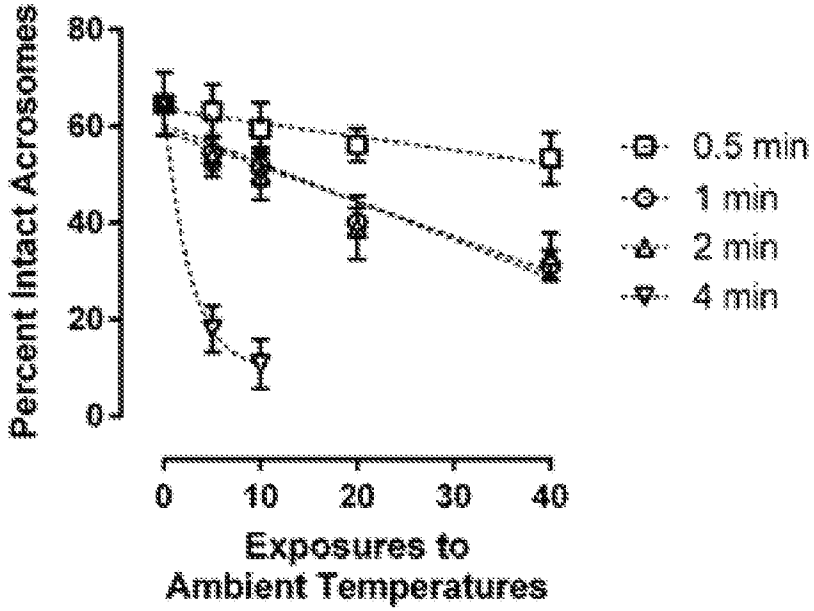

Percent Intact Acrosomes
80
60
40
20
0
0
10
20
30
40
Exposures to
Ambient Temperatures
0.5 min
1 min
2 min
4 min

FIG. 9

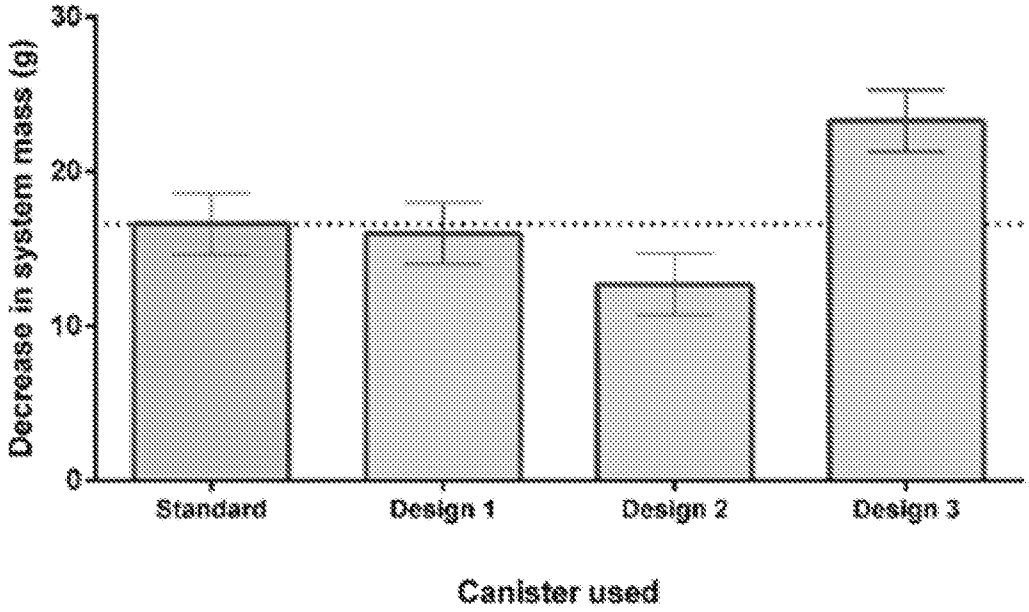

Decrease in system mass (g)
30
20
10
0
Standard
Design 1
Design 2
Design 3
Canister used

FIG. 10

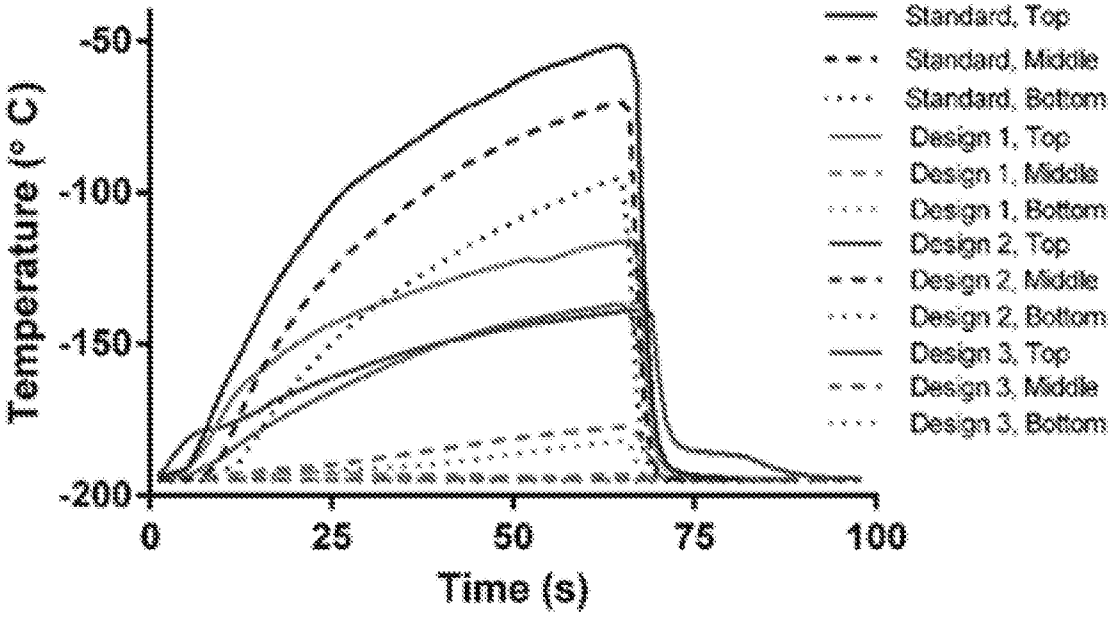

Temperature (° C)
-50
-100
-150
-200
0
25
50
75
100
Time (s)
Standard, Top
Standard, Middle
Standard, Bottom
Design 1, Top
Design 1, Middle
Design 1, Bottom
Design 2, Top
Design 2, Middle
Design 2, Bottom
Design 3, Top
Design 3, Middle
Design 3, Bottom

FIG. 11

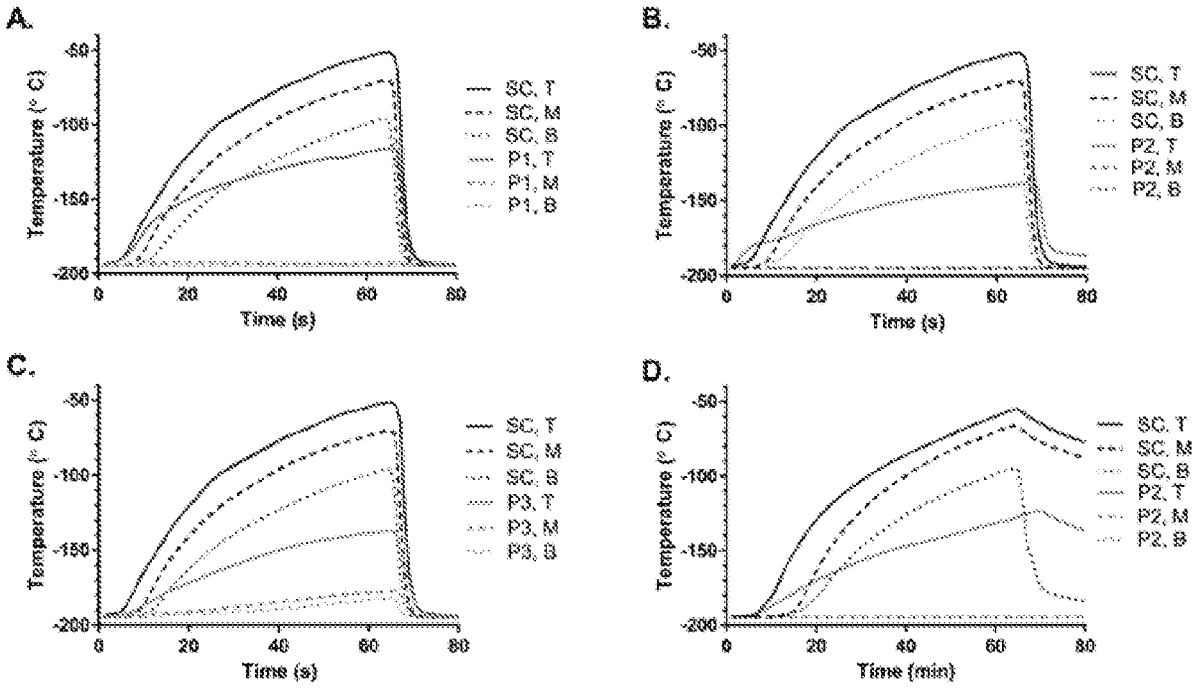
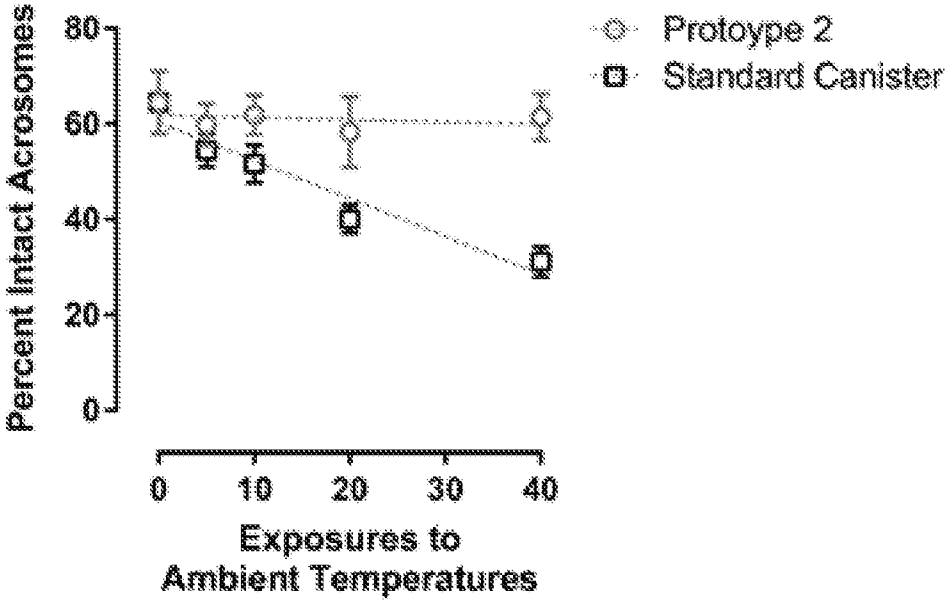

A.
B.
C.
D.
Temperature (°C)
-50
-100
-150
-200
0
20
40
60
80
Time (s)
Time (min)
SC, T
SC, M
SC, B
P1, T
P1, M
P1, B
P2, T
P2, M
P2, B
P3, T
P3, M
P3, B

FIG. 12

12A.



12B.

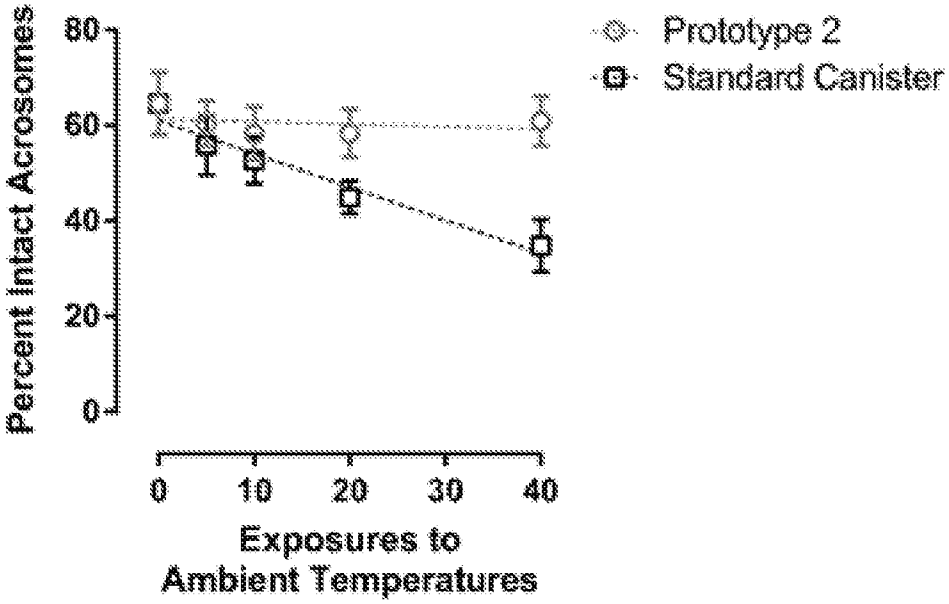
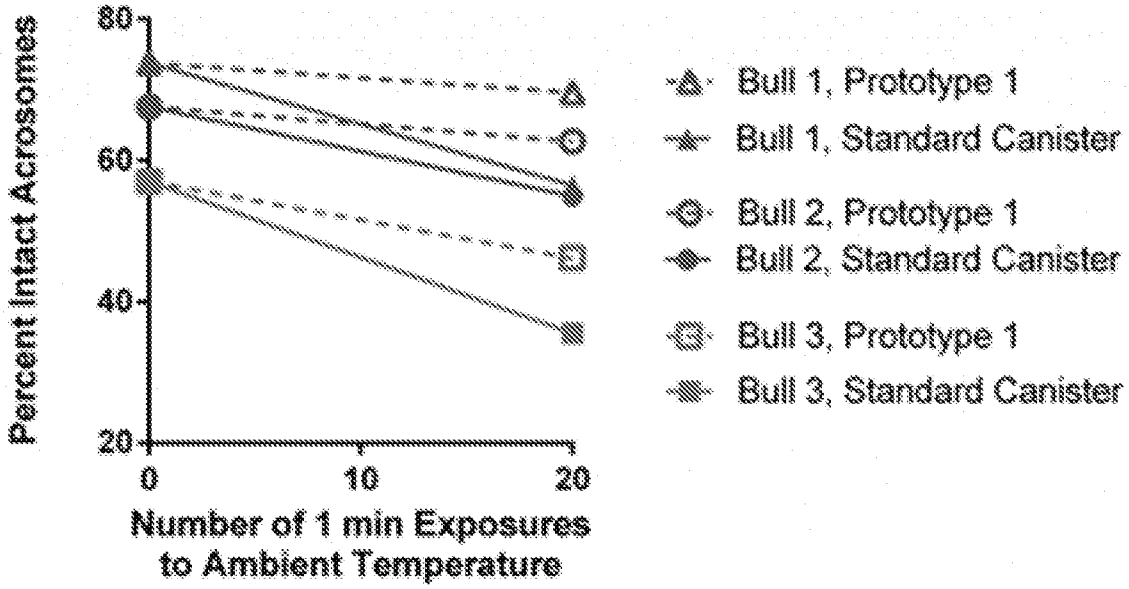


FIG. 13


Percent Intact Acrosomes
80
60
40
20
0
10
20
Number of 1 min Exposures to Ambient Temperature
Bull 1, Prototype 1
Bull 1, Standard Canister
Bull 2, Prototype 1
Bull 2, Standard Canister
Bull 3, Prototype 1
Bull 3, Standard Canister

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/035160

A. CLASSIFICATION OF SUBJECT MATTER

F17C 11/00(2006.01)I, F17C 13/06(2006.01)I, F17C 3/00(2006.01)I

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
F17C 11/00; F25B 19/00; B65D 81/38; F25D 3/12; B65D 1/02; F17C 3/08; F17C 13/06; F17C 3/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: semen straws, container, liguid nitrogen, hole, aperture, and absorbent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011-0056902 A1 (COGNARD, ERIC) 10 March 2011 See paragraphs [0002], [0052]-[0060], and figures 1, 4A-5.	1-2,4-9
Y		3,10-15
X	US 5419143 A (LEONARD et al.) 30 May 1995 See column 3, line 35 - column 4, line 41, column 6, line 51 - column 9, line 23, claims 11-12, and figures 1, 8A-12.	16-23,27-29,31-33,37-38
Y		3,24-26,30,34-36,39
Y	US 3108840 A (CONRAD et al.) 29 October 1963 See column 1, lines 14-19, column 3, line 54 - column 4, line 70 and figures 1, 8-9.	10-15,24-26,30,34-36,39
A	US 2012-0297797 A1 (COGNARD et al.) 29 November 2012 See paragraphs [0022]-[0027] and figure 2.	1-39
A	US 4262494 A (KAROW, JR., ARMAND M.) 21 April 1981 See column 2, line 65 - column 3, line 39, claim 1, and figures 2-3.	1-39

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 September 2016 (07.09.2016)	**07 September 2016 (07.09.2016)**

Name and mailing address of the ISA/KR
International Application Division
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Telephone No. +82-42-481-3360

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2016/035160

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		RU 2454617 C2	27/06/2012
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		EP 2715210 A4	15/04/2015
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		FR 2975749 B1	28/06/2013
		US 8956855 B2	17/02/2015
		WO 2012-162384 A1	29/11/2012
US 4262494 A	21/04/1981	None	