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Cryoprotectants & cryopreservation of equine semen: a review of industry cryoprotectants and the effects of cryopreservation on equine semen membranes

Abstract

Equine semen is a one of the most difficult in the industry to cryopreserve efficiently without causing damage to the membrane or apoptosis. This review consists of an in depth analysis of current cryoprotectant classes, membrane damage issues, reactive oxygen species generation, and apoptosis- all of which are exacerbated by cryopreservation.

Keywords: cryopreservation, equine, semen, membrane, apoptosis, cryoprotectants, stallion, damage

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Abbreviations: AI, artificial insemination; DMF, dimethylformamide; DMSO, dimethylsulfoxide; CP, cryopreservation; CPA, cryoprotective agents; CPO, cryoprotectants; LPO, lipid peroxidation; MF, methylformamide; OS, osmotic shock

Introduction

No one can argue that artificial insemination (AI) has not changed the practice of animal reproduction. Artificial insemination has provided the means of:

i. Taking a single male ejaculate and breeding numerous females,

ii. Transporting genetics without the necessity of the movement of animals,

iii. Limited the risk of injury during mating and

iv. Limited the transmission of disease.

However, early efforts to incorporate AI into production schemes were limited by the functional lifespan of ejaculated spermatozoa. Therefore, widespread the use of AI Cryopreservation (CP) is the ability to store cells and maintain their integrity and viability at a sub-zero temperature until needed. Like some of the greatest discoveries, CP of semen was the result of a fortunate lab error. In 1949, Ernest John Christopher Polge and his colleagues were focused on trying to use sugars as cryoprotectants (CPO) using what they thought was a stock fructose solution.[1] Polge's research was the beginning of a formidable industry, and decades later we are still striving to make improvements.

Discussion

The year 1957 was monumental for CP of equine semen, as Canadians, Barker & Gandier reported the first foaling using frozen epididymal spermatozoa,[2] demonstrating use of the technique was possible in the horse. However, this early success has not led to widespread use of the technique as it has in other industries, due to the unique nature of equine semen. Ultimately, not only will the specificity of the species play a large role, but each individual's own body chemical composition may impact the process as well. Cryopreserved equine semen faces differences in at least two areas: the physiological and biochemical components of the spermatozoa themselves, and variations in the anatomy and physiology of sperm transport in the female reproductive tracts.[3] Currently, stallions generally do not fit the protocols of freezing programs due to the unsatisfactory post-thaw sperm quality and fertility rates.[4] The quantitative differences seen in the required number of spermatozoa necessary for insemination between species is a largely important element when looking at the potential fertility of cryopreserved semen, and if there are a larger number spermatozoa required for insemination this means there can be less tolerance of poor freezers and poor survival rates. In the horse, the accepted number of viable spermatozoa for insemination is more individual dependent than in other species.[3] Studies have consistently shown CP sperm lack in motility, viability and intact membranes when compared to that of fresh semen.[5] Hence, in the horse, the development of successful freezing procedures will involve more than the identification or application of novel CPO's and additives.[3]

Motility remains a major criterion used to determine the success or failure of a new freezing procedure,[2] but this is contradictory since motility does not positively correlate with the sample's future fertility. On the contrary, the classical definition of a "successfully preserved sperm cell" requires they have the ability to fertilize an oocyte, and to produce a viable embryo via AI.[2] Using this definition, frozen/thawed sperm cells must be able to undergo capacitation, activation of the enzymes within the acrosomal cap (while in the female tract), which allows the sperm cell to penetrate through the zona pellucida and fertilize the oocyte.

Cryopreserved stallion sperm exhibits a high degree of male-to-male variability with respect to cell viability after thawing.[4] In order to adequately classify the quality of frozen semen there must be an understanding of the relative classification of a "good" or "poor" freezer. These concepts are based on post-thaw motility characteristics, including percentages of progressively motile sperm and velocity rate.[4] Tischner proved that only about 20% of the stallions exhibit "good" semen freezability with parameters being more than 40% progressively motile sperm post-thaw. "Fair" freezing stallions post-thaw with 60% motility, and progressive motility range of 20-40%. "Poor" quality semen has a <20% survival whereby with a post-thaw progressive motility rate of less than 20%.[4,6]

It is generally accepted that even under the best of conditions that 40-50% of the sperm cell population will not survive CP even with optimized protocols.[7] In some species (including the horse) and specific individuals' survival rates can be much lower, making them self-impeding to use of the CP process. Given the known limitations, Vidament accepted stallions showing a post-thaw motility greater than 35% and sperm exhibiting 'rapid velocity',[4,8] while Loomis & Graham accepted stallions with a post-thaw progressive motility greater than 30%.[4,9] Ultimately, these collaborative efforts have led to the commercially acceptable semen quality post-thaw of 30% progressively motile sperm, however even then there are many stallions that do not meet this standard. Previous studies have evaluated membrane structure characteristics, and suggested, in some cases, there may be genetic detriments, predisposing the cell to certain survival issues under CP stress;[7] supporting the concept of individuals being classified as ''good or ''bad" freezers. By developing an understanding the classes of "good" and "bad" freezing semen, patterns can be established allowing the modification of the components of CPA that permit the further improvement of this area of ART by suggesting which medias are more beneficial for each class and will produce optimal results.

Cryoprotectants

Semen is highly individualistic, as no two stallions have the same chemical composition, and therefore each will freeze differently. Some individuals have been known to be hypersensitive to glycerol, whereas others may be able to tolerate it well. Following industry demands, a wide variety of CPO's as well as many different commercially available CPA's, have been developed. The current consensus is that CPO's work by minimizing exposure to osmotic stress, stabilizing biomolecule and structure, and limit the effects of reactive oxidative species (ROS).[10,11] The ideal CPA would not osmotically dehydrate the cell or induce cryoinjury and it would be non-toxic.[12] The goal of a CPO should be to minimize intracellular freezing, minimize cell damage due to the freezing environment and promote cell survival upon thawing.[7] Larger amounts of CPA concentrations, have be shown to lead to more cellular damage; since cells exposed to those penetrating solutes undergo intense initial dehydration, then rehydration, resulting in a chance of gross cellular swelling to occur when the CPO is removed. Ultimately these radical changes in volume and size can lead to damage and death of the sperm.[13]

CPO Classes

There are two classes of CPOs: penetrating and non-penetrating which when used together, increase the cells' chance at survival while reducing the cellular water content to help prevent intracellular freezing.[7] Penetrating agents are micromolecules, which permeate through the plasma membrane of the sperm cell. Acting intracellularly, penetrating agents replace cellular water, as it pushed to the extracellular region, ultimately preventing internal ice crystal formation that could potentially rupture the membrane. Examples of penetrating agents would include DMSO, glycerol, methylformamide (MF) and dimethylformamide (DMF). Non-penetrating agents or macromolecules, capitalize on their increased concentrations within the extracellular regions during the first phase of freezing, generally -10 to -20°C, where they osmotically extract water from the cells.[7] Some non-penetrating agents worth noting include: egg yolk, sugars, liposomes, milk proteins and polymers that can form extensive hydrogen bonds with water. CPO is generally a combination of penetrating and non-penetrating agents each of which has a specific role in aiding the survival of the sperm cells during the freeze/thaw process. Further discussion of penetrating/ non-penetrating agents with CPA in relation to osmotic stress will follow in a later section.

Glycerol

The used of glycerol as a CPO for stallion semen was first described in 1950, by Smith & Polge. This formulation, remaining virtually unchanged, has be the mainstay of the freezing industry ever since.[14] It has been noted as the most effective CPA for lowering intracellular water freezing[15] while providing osmolality adjustments to the CPO via invasive thermal protection.[16] Research to understand the mechanisms of CPA led to the discovery of glycerol's' effectiveness in its ability to prevent various phase transitions while freezing via increased water permeability and fluidity of the sperm membranes.[17] However, while glycerol has allowed the CP of numerous species, it may not be ideal CPA. Results in cattle have shown a loss of fertility with aged sperm.[16] Further, while glycerol serves as the leading CPO for many species that was not the case for the equine species. While glycerol provides satisfactory protection for the roughly 20% of animals classified as "good freezers," it has proven detrimental to remaining 80% due to its heavy viscosity and molecular weight. Additionally, glycerol has been shown to be toxic to non-frozen sperm and have contraceptive effects on mares.[18]

Initial studies linked glycerol with the stabilization of semen membranes, by its ability to cause a fluid to gel transition, however this finding led to the expectation of higher CP survival rates.[19] However, recent research has demonstrated glycerol induces cellular damage during the freezing process and, in addition to cryoinjury,[19] could be a largely contributing factor to poor post that motility and fertility rates.[14] While the nature of semen glycerol toxicity is not fully known, some data suggests its use may lead to protein denaturation, directly altering the plasma membrane and the disrupting actin interactions.[14] Further, even though glycerol is a penetrating agent, it is extremely slow in permeating the plasma membrane, which induces osmotic stress which may be the ultimately cause of its toxicity. A number of studies have demonstrated that the addition and removal of glycerol is an important factor responsible for the reduction on post-thaw motility and viability of horse sperm. Equine spermatozoa have been shown to have a limited osmotic tolerance. Glycerol has been shown to induce more distinct osmotic stress with more severe alterations on motion variables, cell viability and acrosomal integrity.[14]

The issues with glycerol toxicity have led to the research and testing of countless other penetrating CPA's, with the idea of finding one that will be less toxic, while yielding comparable or better quality results.[20] This new era of CPO bases include combinations of penetrating CPA, for example glycerol and dimethylformamide. Early

results suggest these combinations have lower molecular weights, increased water solubility and minimal toxicity,[14] all of which have proven to advantageous to the preservation of the delicate chemical composition and plasma membrane structure of stallion semen.

Dimethylsulfoxide

DMSO is a sulfur containing, organic molecule, which easily crosses cellular membranes. The fast penetrating capacity of DMSO helps to decrease the amount of time necessary to displace water from the intracellular fluid to the extracellular environment. Given the variability seen in stallion sperm, a small amount of this strong compound is often used in conjunction with glycerol or another CPA as an added speed component, and to help stabilize the cell prior to freezing. However with some species DMSO is favored, and used in much larger proportions than necessary for livestock. Species, whose semen specifically perform better following freezing with DSMO may do so because glycerol acts as a contraceptive for them. A few species that have benefited from DMSO as a primary CPA include: mice, rabbits[21] and a variety of fish-including: zebra fish,[22] carp broodstock,[23] seven-band grouper[24] and mutton snapper.[25] Rabbit sperm appear to do best with a substantial proportion of DSMO in relationship to glycerol for CP. Some research suggests this could be do the lack of water channel protein Aquaporin 7 (AQP7), which coincidentally serves as a glycerol transporter.[21] DMSO has also been used for semen CP in some members of non-human primate family. Two species, both part of the macaque family: Cynomolgus monkey (*Macaca fascicularis*)[26] and Rhesus Monkey (*Macaca mulatta*),[27] have shown mixed results. The Cynomolgus semen was successfully frozen when DMSO was in an equal concentration to glycerol,[26] whereas the Rhesus was unsuccessful after using a stair step increasing trial of DSMO to glycerol.[27]

Amides

Amides have proven to be a mostly beneficial CPA, having been shown to decrease damaging results compared to those obtained when glycerol is solely used as the CPO.[28] With stallions being sorted into different freezing classes, poor freezers have required substantial work. Amides have increased the freezing potential of this class while subsequently decreasing the overall damaging results induced from glycerol.[28] While glycerol is still the main CPA used for stallion sperm, the addition of amides, in part due to their lower viscosity and molecular weight, may decrease sperm cell damage.[29] DMF has been shown to enhance post-thaw motility, preservation cellular membranes which may effectively enhancing semen freezing potential.[19] With lower molecular weights, both DMF and MF are able to permeate stallion sperm, more efficiently than glycerol, which has resulted in reduced swelling during equilibration in amide-containing diluents and not as toxic as glycerol.[28] However, DMF and MF seem to be the only amides with possible cryogenic effects. Studies have shown that other amides have detrimental effects on semen not being cryoprotective.

As previously discussed, stallion sperm is highly individualistic, because of this; research into additional CPA's that would help to preserve frozen semen has flourished. The discovery of MF and DMF as agents has been more than beneficial to the industry and spurred investigation of other alternative agents. Reductions of freezing and thawing damage, improving membrane integrity and increasing progressive motility have been the goal of equine cryobiology researchers over the last 30years. During that time, reducing damage due to freezing and thawing has been the focus of most investigating alternative CPA's. Recently, work with the addition of liposomes, which induce fusion to the sperm plasma membranes, as well as the reversible binding of exogenous phospholipids, have both shown to protect sperm from damage.[30] Cholesterol and methyl-β cyclodetrin[31] have been shown to reduce membrane transition temperatures, resulting in reduced cryoinjury, maintenance of the cellular membranes and improve post-thaw motility. Like most potential CPA, the use of a lipid based CPO has been demonstrated to have both positive and negative effects on equine semen.

Lipids

Previous studies have demonstrated CP can lead to loss of from the membrane leading to peroxidation and continuing on to form reactive oxidative species.[32] Lipid bases are multifaceted since they have been linked to both oxidation of, as well as the protection of lipid bilayer infusions. The addition of a lipid based CPO may destabilize the sperm membrane due to the formation of ROS and recruitment of lipids from the membrane leading to lipid rearrangement within the membrane itself causing additional oxidation to occur. Increased peroxidation in turn might affect both motility and acrosomal activity. Sperm are prone to cold shock damage due to osmotic stress and relative temperatures which in turn may lead to underlying damage to the integrity of membrane. Further studies are need to determine the exact role lipids play in protecting spermatozoa during freeze-thaw is unclear.[32] Therefore if lipids are to be added as a cryoprotective agent to produce a more saturated CPO for semen preservation, there are a few other issues which must address. Numerous studies have shown that ROS play a significant role in male infertility.[33] Further, cold shock damage has been directly linked to lipid phase transitions that cause the sperm membrane to become leaky, thereby compromising membrane integrity.[32] However, ROS been shown a double-edged sword. While their detrimental effects are well documented, at low levels they are involved in the normal physiological functions of sperm including capacitation, acrosome reaction, and binding to the *Zona pellucida* at physiological concentrations.[33–35]

Optimizing formulas

While the usage of amino acids with stallion sperm has not been studied extensively, the few studies done today suggest they may be an important addition to extender formulations. Koskinen et al.[36] demonstrated the addition of betaine, as a stallion CPA, which stimulated increased post-thaw motility.[36] Initial testing from Sanchez-Partida et al.[37] working with frozen ram sperm demonstrated low concentrations of proline, glycine and betaine could be used to improve post-thaw motility as well.[37] Trimeche et al.[38] also showed that low concentrations of proline to be beneficial in enhancing the motility parameters of stallion sperm. Glutamine has been helpful when combined with glycerol for human sperm post-thaw motility and viability,[39] and at low concentrations, it has worked effectively in stallion semen.[38] Conversely, high concentrations of betaine, glutamine, histidine and proline were demonstrated to cause significant dropped in sample motility.[38] As mentioned above, work has been done with other amides as well. However, unlike the beneficial effects described for MF and DMF, acetamide, and formamide[18] both appear to be toxic to stallion semen, and have poor cryoprotective properties which make them unsuitable as a CPA.[40]

Membrane issues

Baird's Tapir are evolutionarily related to equids and rhinoceros.

Tapir's semen osmolality has proven similar to that of the domestic horse, Prezwalskis horse, and the rhinoceros.[41] However, the average pH of the samples being lower than the three aforementioned species, the difference can be attributed to accessory gland contributions.[41] Due to the shared evolutionary relationship between these species, the industry knowledge that has been acquired for stallions may help to determine appropriate CP techniques and CPA that may be applicable for the other equid species. With stallions, it has been proven that the addition of cholesterol helps to increase the spermatozoa permeability to CPO thereby increasing the osmotic tolerance, and improving the sperm cryosurvival rates.[41] Given the semen osmolality similarities it has been suggested that cholesterol be included, to help facilitate better Tapir semen preservation.[41]

One particular challenge for the Tapir, as with any non-domesticated specie, is the collection of the sample. While domesticated stallions are able to be collected via an artificial vagina, this is an unrealistic approach to nondomestic species, as Tapirs would be more at risk to injury.[41]

Prezwalskis' have not been cryobiologically studied, and therefore currently rely on research information gather from the domestic horse, especially concerning sperm cryosensitivity.[42] It is a well-established fact that less than 20% of domestic stallions produce sperm that are capable meaningful post-thaw survival, mainly due to the variations in individual CP capacities. This appears to be the case with the Prezwalskis as well. As in any species, there is the challenge of minimizing toxic CPO impacts are vital. However, there is evidence that amides will help to mitigate toxic impact of these compounds.[42] Current research has suggested that Prezwalskis spermatozoa are tolerant of cryoagents, cryodilutents, and the processing used in the domestic horse industry. These findings give hope for the potential of CP of a species that is facing extinction.[42]

Cryonjury

Mazurs'[43] two factor hypothesis on freezing injury, helps to categorize and explain the freezing responses from different cell types. The osmotic behavior of cells is widely understood, respectively with each species and cell type, having unique boundaries. However, the increased variation between stallions compared to other species, make this less predictable. Recently, it has been shown that rapid cooling of human and stallion sperm infers a loss of viability, but more interestingly suggested that intracellular ice may not be the culprit.[44] Demonstrating how speeding up the cooling rates, Morris et al. would reduce the ice crystallization damage by "solution effects." They also suggested that those same higher cooling rates are found in glycerol solutions. Given what we know about stallion sperm, this relationship appears counterintuitive, as the majority of glycerol based CPO's have been shown to have a more deleterious effect on sperm cells. Morris also suggested that post-thaw semen quality might be just as dependent on semen concentration as well as any single CPA of the additive.

Finally, in addition to the biochemical and physiological issues above, both the methodologies used for collection as well as the mechanics preparing sperm for freezing may result in significant cell loss. While many techniques are more than suitable for producing a fertile sample, the loss of semen is inevitable and can be classified into the following: loss during receptacle transfer, loss in centrifuge tubes, loss due to air exposure,[45] with cautionary techniques used, there is still loss involved, most of which can be attributed to extended air exposure. Stallion sperm can use both aerobic and anaerobic pathways[46] and with fluxing temperature and air exposure, the concept of motility conservation minus air exposure results in the decline of energy stores via glycolysis and glycogen recruitment.

A basic understanding of the architecture of the sperm membrane is crucial to understand how cryoinjury occurs to cells. Nowhere is this more true and with more impact on fertility than damage to the acrosomal membrane. Membrane composition and fluidity of the individual lipid bilayer is highly dependent on the dietary intake.[47] The intercalation of CPO or other compounds affect membrane fluidity, cause changes to the cytoplasmic viscosity, ultimately affecting the cell's metabolic capacity. Concurrently, when cells are introduced to low temperatures that they would not normally physiologically encounter, the membrane alters its mechanism of lipid packing, which modifies enzymes within the membrane and the kinetic properties of the cells. All of these factors lead to the imminent potential of cryoinjury, including: cold shock, freezing damage or thawing damage.[47]

Apoptosis

Apoptosis can occur within all cells, resulting in programmed cell death. The physiological process of programmed cell death which affects single cells and induces morphological and biochemical changes which lead to cell death and acts as a homeostatic function within the body. It has been shown to occur within spermatogenesis as a homeostatic event, to help balance the new and old cells. Since apoptosis is required to allow the normal development of germ cells, spermatogenic apoptosis helps to maintain the balance between germ and somatic cells, while also removing the defective germ cells.[48] However if this process is interrupted it could lead to increased quantities of ejaculates spermatozoa displaying apoptotic like changes and result in decreased fertility. With a two-pathway option for apoptotic initiation, the intrinsic is due to pre-apoptotic signals that lead to the activation of caspases, and extrinsically death receptor pathway receptors allow for ligand binding to occur at the plasma membrane again leading to activation of caspases. The current two theory methodology for apoptosis include abortive apoptosis which is the marking of defective germ cells during spermatogenesis, but instead of apoptosis occurring, they are able to escape the testes. The second theory is mature ejaculated spermatozoa are undergoing apoptosis or an apoptosis like process; initially this was thought to have not occurred, but recent studies have shown that ejaculated sperm are capable of triggering caspase activation.[48]

Spermatozoa are exposed to a variety of physical and chemical stresses during CP, changing the lipid composition of the plasma membrane, head size as well as resulting in DNA damage[49] and increased plasma membrane lipid disorder[20] allowing the supposition that apoptotic like changes may be induced in equine sperm CP.[48]

A portion of the cell loss that occurs during CP as cells already programmed to die are included in the freezing process and may reduce the number of viable cells in an AI dosage. Moreover some of the more subtle damage that is caused to sperm cells via CP may help to induce this programmed cell death, and therefore lower viability numbers transferred and/or reduced life span of those cells when in the female reproductive tract. The apoptotic phenomena[50] of cryopreserved stallion's sperm is attributed to oxidative stress, phase transitions of the plasma membranes, cryocapacitation, as well as the premature activation of the pathway due to subtle damages.

Unfortunately, without extensive testing, there is no easy way to determine a cell that has begun the apoptotic process. These defective cells are programmed for removal, but unfortunately with only one pathway out, the expulsion of dead cells occurs consistently within the ejaculate.

A cause of apoptosis normally overlooked during semen processing is the presence of microbes in the semen sample.[19] Results from a more recent study has shown that stallions ejaculate is more in line with that of humans due to the bacteria which induce sperm apoptosis[51] and necrosis;[52] with the microbial flora playing a critical role in the sublethal apoptotic damage that stallion spermatozoa experience during CP and cooled storage.[53]

Reactive oxygen species

A recent set of studies looked the activity of proteins, apoptosis and ROS, the proteins involved in the activation of apoptosis and the inductor protein involved in the activation of the mitochondrial pathway of apoptosis-have been found in fresh, frozen and thawed equine spermatozoa.[54,55] Together these studies support the idea that ejaculated spermatozoa can trigger activation the nuclear matrix potentially leading to cleavage of the entire sperm DNA into small fragments.[56] There is still controversy about the apoptotic markers that have been found in equine semen subpopulations and if this information is actually a significant, subsequent information collected post CP would need to be done for analysis for equine semen.[28]

Further, depending on training of individuals involved, counts may include no sperm cells such as residual bodies. Residual bodies are made from cytoplasmic portions of elongated spermatids,[50] and are subsequently shed with viable sperm cells into the seminiferous tubules, and therefore into the ejaculate.

Osmotic shock (OS) has long been associated with and a major factor in sperm damage during cryopreservation; and while this statement still holds true, newer research demonstrates it is just one potential problem. The influx of hypertonic concentrations while freezing and the hypotonic concentrations when thawing have been shown to induce OS which has been shown to be detrimental to the integrity of sperm cells. Somatic cells have been well documented to show that OS is responsible for apoptosis, cell cycle arrest, DNA damage, oxidative stress as well as a variety of other actions.[29]

This is especially true in stallions, as spermatozoa have a very limited osmotic threshold[57] which ultimately results in uncontrollable shrinking and swelling of the sperm head causing damage to the semen. Studies have shown that stallion sperm damaged during flash freezing and morphologically abnormal sperm generate greater amounts of ROS.[58] While the fluidity of the plasmalemma is able to tolerate and adjust to these changes without penalty, OS may lead to a loss in viability, poor motility, and/or a decrease in the mitochondrial membrane potential.[59]

Peroxidation of plasma membrane lipids (lipid peroxidation, LPO) has been proposed to be a major factor involved in sublethal cryodamage of sperm in many species, including horses.[58] Two pathways may result in the formation of LPO's; the enzymatic membrane system using NAD(P)H as a substrate and the mitochondrial electron transport chain. The main source of oxidative stress for spermatozoa is the mitochondria.[60] ROS production is increased in sperm mitochondria due to freezing and thawing, while an osmotic mechanism may increase mitochondrial membrane permeability, thus activating apoptosis. In fact, oxidative stress is a well-documented inductor of apoptosis.[58] The concept of relative osmotic stress to the hyper and hypotonic environments has been shown to define the range, which may represent the "osmotic tolerance limit." Once these limits are surpassed, they cause irreversible damage to the cell, preventing the spermatozoa from recovering its initial motility when returned to isosmolality environment. Pommer et al. suggested this hypertonic limit for motility was reached at 450mOsm/kg[61] while Garcia et al. demonstrated a hypertonic effect at 1500mOsm/kg, which prevented stallion spermatozoa from recovering their initial volume once osmotic balance was restored.[29]

Conclusion: the future of semen cryopreservation

As should be apparent from the forgoing material, CP of equine semen is desired by the industry as a means of long-term preservation and storage of superior genetics. While the vast majority of the research has focused on CPO's, there remains a need for a simpler device that is able to provide the same quality results, which we are currently obtaining from the programmable freezers. Equine CP, has be something which has been more than problematic for the industry, and just within the past ten years we have broken through to new methods which are allowing us to achieve the idea of a superior yet simple device. Programmable freezers using electricity have been the detriment to stallions, and the poor post thaw recovery rates generated from vertical mist have pushed the industry forward. The reduced quality of semen post-thaw is a clear response to the sub-optimal cryopreservation protocols that are in use, as the majority of cellular damage has been reported to occur between in the initial freezing stages.[62] Ideally an optimal freezing rate must be slow enough to prevent intracellular ice formation, but fast enough to avoid cryoinjury.[62] The necessity for a simpler device has been acknowledged and the equine reproductive industry has begun to explore alternative options. With sperm being susceptible to rapid cold shock injuries especially during the initial process and leading to membrane damage, the goal of this study was to slowly yet effectively control and decrease the temperature.[63]

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Conflict of interest

Author declares that there is no conflict of interest.

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