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Effects of delivery time and different cryopreservation methods on canine epididymal sperm

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Table of content

1 Introduction

Cryopreservation, the process of preserving biological cells at ultra-low temperatures, is gaining importance in various fields in recent years. The primary goal of cryopreservation is the maintaining of the viability and function of these biological materials, such as oocytes, embryos or spermatozoa, over long time. This allows the storage for future use or scientific research. The principles of cryopreservation are (1) the storage at very low temperatures, (2) the use of cryoprotectants for the prevention of ice crystal formation in the frozen cells, which lead to cell damage and (3) the storage in liquid nitrogen at -196°C, to maintain the stability of the preserved material. There are numerous areas of application, such as the biomedical research, the preservation of the fertility in human and veterinary medicine and the genetic preservation. In this regard, in assisted reproduction techniques cryopreservation is commonly used. Regarding the veterinary medicine, in the pig and cattle industry the usage of cryopreservation techniques is part of the daily routine. The artificial insemination with frozen-thawed semen is a safe and accurate method to ensure optimal fertilization rates. There are multiple cryopreservation techniques developed over time. Every method has its own characteristics and implementations, which are resulting is different advantages and disadvantages.

The focus of this study was the cryopreservation of epididymal semen in dogs. The preservation of dog semen in general has a lot of advantages. An azoospermic ejaculate or a “dry ejaculate”, as it is called in case of aspermia, might not be the end of the hopes of procreation. A donor male that accidentally dies, is euthanized or undergoes orchiectomy for medical reasons can still generate offspring. In both cases, the precious germplasm can be hidden in the epididymides as a treasure with great potential. Epididymal spermatozoa can be retrieved from ex vivo or in vivo testicles, can be cryopreserved and used in assisted reproductive technologies (ARTs) [1].

The global distribution of frozen dog semen brings the possibility to ensure the preservation of a wide genepool in rare breeds. Recent years have seen a drastic decline in biodiversity. In addition to the traditional in vitro conservation of genetic material, there is also a need to establish a well-functioning protocol to cryopreserve the genetic material of a male of an endangered species or with valuable genetics. Storage of

epididymal sperm is a feasible method to achieve this goal, however, time of sample delivery and freezing technique are key factors affecting the success rate.

2 Literature review

2.1 Cryopreservation: basics and relevance

The viable freezing of biological material and their long-term storage at ultra-low temperatures, typically at or below -196°C using liquid nitrogen, is called cryopreservation [2].

In recent years, there has been a steady rise in the effectiveness of preserving cells and tissue through cryopreservation, thanks to the utilization of cryoprotective substances and advanced temperature control devices [3].

Cryoprotectants are added to the biological material. In general, there are two types of cryoprotectants: membrane permeating and non-membrane permeating cryoprotectants. The permeating ones can freely diffuse into the membranes. The capacity of these structures to engage in hydrogen bonding with water significantly contributes to their protective functions. Typically, when water undergoes freezing, the emerging crystalline arrangement expels dissolved substances as it establishes its lattice. These substances are pushed into the dwindling liquid phase, causing a rise in their concentration to potentially lethal levels within the cell. However, due to the robust hydrogen bonding interaction between permeating cryoprotectants and water, the freezing point of water is lowered. Consequently, fewer water molecules are accessible for self-interaction, hindering the creation of crucial nucleation sites needed for crystal formation [4]. Example for this type are glycerol, ethylene glycol and dimethyl sulfoxide [5].

The non-membrane permeating ones cannot permeate the cell membrane. Polyethylene glycol, polyvinylpyrrolidone, raffinose, sucrose, and trehalose are examples for this type of cryoprotectants [6] [7].

The cryoprotectants help to minimize the formation of ice crystals to prevent cellular damage during the freezing and thawing process. A milestone for the development of cryopreservation techniques was set in 1949 when Polge et al discovered that glycerol had a significant cryoprotective effect [8]. Glycerol is often used in the cryopreservation of animal spermatozoa [9].

Cryopreservation techniques are highly relevant in the fields of animal conservation, research and breeding.

Clinical applications of cryopreservation include the preservation of male fertility before radiotherapy and/or chemotherapy, the use of frozen partner and donor spermatozoa for artificial insemination (AI), and in vitro fertilization (IVF) in infertility treatments. Furthermore, the ability to successfully cryopreserve epididymal and testicular sperm samples is important and useful for treating patients with obstructive and nonobstructive azoospermic who require the use of assisted reproductive technologies (ARTs) [10].

Regarding the conservation of endangered and threatened species, cryopreservation plays a crucial role in preserving the genetic biodiversity. The preservation of genetic material from endangered species through cryopreservation techniques helps to prevent species extinction and may support future reintroduction efforts. Agca and Critser mentioned that the use of frozen-thawed gametes from rare and endangered species in assisted reproductive technologies will gain greater significance within conservation biology initiatives [11].

Cryopreservation techniques are essential in scientific research, including studies related to reproductive biology, genetics and the development of assisted reproductive technologies [3] [12]. Furthermore, researchers can conduct experiments without the need for live animals by using cryopreserved samples.

As mentioned before, cryopreservation is widely used in assisted reproductive technologies.

In dogs there are different available assisted reproductive techniques. The possible artificial insemination (AI) methods are the trans-vaginal, the trans-cervical and surgical insemination using either fresh or frozen semen [13] [14]. During the trans-vaginal AI, the semen is placed into the vagina with the help of an insemination pipette. The trans-cervical technique can be performed using the Norwegian or New Zeeland endoscopic technique. In both techniques the spermatozoa are deposited directly into the uterus, after manipulating the needed catheter through the cervix [13].

During the cryopreservation process, cells are exposed to cryoprotective agents such as glycerol, ethylene glycol or dimethylsulfoxide in an extender. The addition of ‘cryoprotectant’ agents exposes the cells to a hypertonic environment; this causes the cells to initially shrink, but they regain their normal volume as the cryoprotectant enters the cells. Cooling also changes the cell membrane. During freezing, cells again shrink as water flows out of the cell and ice forms in the extracellular space. Depending upon the cooling rate, ice may form inside the cell as well. Formation of intracellular ice usually results in damage to the cell membrane; it loses its semi-permeable properties, resulting

in death of the cell. During thawing, the cells undergo similar changes in volume as water moves back into the cell. The thaw rate and cell survival are dependent upon the cooling rate used. As stated by a researcher considering the difficulties in semen cryopreservation of other species compared to the bull, 'it causes one to marvel at successful survival rather than to despair over the difficulties of its implementation to the preservation of a variety of other sperm cell types' [15] [16].

Canine semen cryopreservation methods can be grouped into three main categories: slow (programmable) freezing, ultra-rapid freezing and vitrification. The programmable freezing provides a machine-controlled cooling system with accurate cooling rates, and multiple steps of equilibration. However, the veterinarian/operator needs to purchase a relatively expensive equipment. Ultra-rapid freezing requires only a styrofoam box, which is partly filled with liquid nitrogen. The samples are put 4-6 cm above the nitrogen, resulting the cooling period in nitrogen vapor. Due to the cost-effectivity, this technique is the most widely used in the daily routine, however, uncontrol cooling rate can be controversial. In recent years, there are increasing numbers of data on the application of vitrification in canine semen freezing. In this case, the diluted samples are collected in pipettes and droplets are added directly to the liquid nitrogen. The methods provide extremely high cooling rates, which can be advantageous for post-thaw viability [17].

2.1.1 Semen cryopreservation

Semen cryopreservation is a routine assisted reproduction technique in dog breeding [18]. In the literature there are multiple reasons mentioned why the sperm cryopreservation is of such a high importance, including: preserving the genetic variety within populations of both domesticated and wild canine males [19] [20], promoting the spread of domestic species lines that exhibit superior genetic traits and storing genetically altered animal models that mimic human health and disease in a genetic repository [20] [21].

Cryopreservation of gametes is gaining more and more attention among biologists, clinicians and pet owners. Aimed to preserve fertility or to widen the diffusion of specific genotypes in case of single valuable animals, or to safeguard biodiversity in case of endangered breeds or species, techniques and protocols to store germplasms from domestic and wild species have been developed over the years.

Cryopreservation of male gametes is probably the most efficient among reproductive cells. Spermatozoa are small, but have a large surface/volume ratio, and these features make them quite easy to freeze and scarcely prone to suffering cryoinjuries. In dogs, sperm slow freezing is getting more and more used, since it allows breeders and owners to obtain puppies from long distance living animals [22].

2.1.2 Epididymal sperm preservation

Epididymal semen preservation is a procedure used to collect and store the sperm from the epididymis. The epididymis is a long, coiled tube located on the back of the testicles where sperms are matured and stored. This technique is used for various purpose in science and canine reproductive medicine, such as artificial insemination and for preserving the genetic material of high value males. This procedure can be performed in cases of sudden death of genetical highly important males [3].

The tolerance of epididymal sperm to cold temperatures appears to vary compared to ejaculated sperm, with epididymal spermatozoa being regarded as more vulnerable to cryodamage [23] [24] [25] [26] [27] [17]. For example, a notable reduction in sperm mobility is evident within just a 2-hour equilibration period at 4°C [26]. Beyond the inherent variability among dogs regarding the freeze-thaw capability of their epididymal sperm [28], there is frequently a marked decline in sperm viability and membrane integrity. This includes alterations in the plasmalemma and the premature occurrence of the acrosome reaction upon thawing. The limited ability to withstand cold temperatures in sperm might be attributed to variances in both structure and function between sperm found in the epididymis and those ejaculated. These distinctions could stem from the presence of the cytoplasmic droplet or the absence of exposure to prostatic fluid. The migration of the cytoplasmic droplet in dogs takes place within the epididymal corpus [29]. This droplet is situated near the annulus at the distal end of the midpiece in the sperm cells obtained from the cauda.

The droplet could potentially cause a decrease in the flexibility and stability of the membrane, which in turn may undermine the sperm's ability to withstand freezing temperatures. There is some disagreement in the research findings when it comes to how prostatic fluid affects sperm. However, it has been demonstrated that when added to the preservation solution prior to freezing, prostatic fluid enhances the post-thaw motility and

acrosomal integrity of epididymal sperm (Hori et al., 2005). Their longevity is increased during the incubation after thawing [25].

In contrast other researchers reported that while prostatic fluid initially enhances sperm motility, it exerts an adverse impact on chromatin integrity, with a delayed onset occurring four hours following the thawing process [30].

The underlying process responsible for the beneficial impact of prostatic fluid remains unclear. It is conceivable that glycoproteins present in prostatic fluid, which encompass decapacitation agents, might envelop the sperm's exterior, safeguarding acrosomes against cold-related harm. Furthermore, prostatic fluid could potentially obscure progesterone receptors that are involved in the acrosome reaction of sperm cells [31] [32].

An alternative explanation relates to its antioxidant composition. It's widely recognized that sperm cells are highly sensitive to oxidative stress, primarily because they have fewer antioxidants in their reduced cytoplasm and possess a plasma membrane rich in polyunsaturated fatty acids. Unlike seminal plasma, epididymal semen lacks the protective influence of antioxidants, making it potentially more susceptible to oxidative stress during the cryopreservation process.

While the canine epididymis does exhibit antioxidant activity, the inclusion of prostatic fluid may enhance the defense against the detrimental impacts of reactive oxygen species and lipid peroxidation [33] [34].

Regarding the in vitro fertilization capacity of the sperm from different origin, controversy results were made. When utilizing spermatozoa from the epididymis for in vitro embryo production, the outcomes were notably poor [35] [36]. Considered that the outcomes of in vitro fertilization with canine oocytes exhibit significant variability and low success rates, even when utilizing ejaculated sperm cells. This is predominantly due to the inefficiency of the in vitro maturation process of the oocytes. Controversy, artificial insemination has reached positive results. Fresh and chilled epididymal sperm used for intravaginal insemination, led to the birth of eight puppies and one puppy [37] [38].

Several healthy puppies were successfully delivered after cryopreserved epididymal spermatozoa were used for surgical intrauterine or intratubal insemination [39] [40] [41] [42].

Although there have been certain developments in this area, the anticipated enhancement of in vitro embryo production in dogs through techniques like in vitro fertilization or intracytoplasmic sperm injection is expected to significantly expand the possibilities for utilizing epididymal spermatozoa in this species.

2.2 Semen examination methods

Semen analysis offers insights into the process of sperm production and the capacity of a specific male to successfully fertilize [43]. Every semen examination has to evaluate of the concentration, motility and morphology of sperm cells [44].

2.2.1 Motility analysis

The computer-aided sperm analysis (CASA) is nowadays routinely used as a semen examination method regarding the motility evaluation [43]. The subjective and conventional motility analysis can vary between operators, because of variation regarding their training, skills and experiences. Differences ranging from 30% to 60% have been documented when subjective evaluations are used to assess the motility characteristics of identical ejaculate samples [45]. Regarding this, it was important to implement a more objective and accurate evaluation system [46].

2.2.2 Morphology analysis

The morphology analysis of the spermatozoa is done by microscopic analysis. Stained preparations, the oil-immersion brightfield objective, with 100x magnification, and at least an eyepiece with 10x magnification is used for this purpose.

The recommended classification is a simple normal/abnormal one. The tallying of the location of the abnormalities in abnormal spermatozoa can vary from study to study.

The body of the Spermatozoa in general include a head, neck, midpiece, principal piece and end piece. Regarding the difficulty to see the end piece with a light microscope, the spermatozoa can be separated in a head (and neck) and tail (midpiece and principal piece).

A cell is seen as normal in the case both head and tail show no abnormalities.

Normal heads have a regular contour, are smooth and their shape is oval [47]. 40-70% of the head should be a well-defined acrosomal region [48].

The midpiece should be as long as the head of the sperm cell, regular and slim.

There can be several possible morphological abnormalities seen in the analysis:

Head defects can contain: Micro- and Macrocephalus (small and large heads), acrosomal area can be small or large, tapered, pyriform or round head, double heads or any combination of these.

Neck and midpiece defects can be noted: Thick or irregular, thin, insertion of midpiece into the head is asymmetrical or any combination.

Principal piece abnormalities can be the following: short, numerous, fragmented, smooth hairpin curves, sharply angulated turns, of varying width, coiled or any combination of these defects [47].

In this study the abnormalities were analyzed and summarized in the categories of acrosome defect, detached head cells and tail defects.

2.2.3 proAKAP4 analysis

AKAP4 is synthesized as a precursor protein named proAKAP4 and the release of a sequence peptide called prodomain provides the mature AKAP4 [49]. Both AKAP4 and proAKAP4 are found in mature sperm [50] [51] [52] and proAKAP4 has been considered an objective sperm quality and fertility biomarker [50] [53]. In humans, AKAP4 is clearly downregulated in infertile patients with asthenozoospermia [54] [55]. Evidence of the role of AKAP4 in bull fertility has increased over the years [50] [56] [57] [58] [59]. AKAP4 transcripts were identified among the top five validated transcripts, and significantly increased in high compared to low quality bull semen [57]. By testis transcriptome profiling, the AKAP4 gene was found to be drastically downregulated in sterile hybrid male cattle yaks due to spermatogenic arrest [58]. The proAKAP4 concentrations were recently described to be correlated with the total and progressive motility of spermatozoa in bulls [60]. Recently, this protein was found in canine spermatozoa [61].

CASA is the gold standard in sperm motility assessment. However, the equipment requires huge financial investment of the laboratory. ProAKAP4 measuring kit can be a less expensive, feasible alternative for automated motility assessment.

3 Material and Methods

The objective of our study was to investigate the freezing ability of canine epididymal spermatozoa in fresh stadium and after 24 hours storage at 4°C with two different freezing protocols.

3.1 Experimental design

Testicles from 10 dogs were collected during orchiectomy in the Small Animal Clinic for Obstetrics at the University of Veterinary Medicine Budapest. The sample of each dog was cryopreserved with two different freezing protocols at different times.

Spermatozoa were collected from the tail of the epididymis. The sample of one testicle was cryopreserved immediately after harvesting in fresh stadium, whereas the second testicle was stored for 24 hours in phosphate buffered saline at 4°C before the freezing process. Each sample (fresh and 24-hour storage) was cryopreserved by using the ultra-rapid and the vitrification method.

Consequently, this results in 6 treatment groups:

- **Fresh 0h:** Non-cryopreserved, fresh group. Motility and morphology analysis were performed immediately after the sample was collected.
- **Fresh 24h:** Non-cryopreserved, fresh group. Motility and morphological analysis were performed after 24 hours of incubating 4°C.
- **UR 0h:** Samples were cryopreserved with ultra-rapid freezing method immediately after the sample collected. Motility and morphology analysis were performed after thawing.
- **UR 24h:** Samples were cryopreserved with ultra-rapid freezing method after 24 hours of incubating 4°C. Motility and morphology analysis were performed after thawing.
- **VF 0h:** Samples were cryopreserved with vitrification technique immediately after the sample collection. Motility and morphology analysis were performed after thawing.
- **VF 24h:** Samples were cryopreserved with vitrification technique after 24 hours of incubating at 4°C. Motility and morphology analysis were performed after thawing.

Following parameters were evaluated immediately after harvesting, after 24 hours of cool storage of one testicle at 4°C and post-thawing: total and progressive motility, normal morphology rate and abnormal morphology rate, such as rate of acrosome defects, rate of detached heads and rate of tail defects. Furthermore, the proAKAP4 concentration was evaluated via ELISA test for all treatment groups.

3.2 Animal origin and sample collection

The dogs involved in the study all came from several animal shelter around Budapest. They are mixed breeds and aged between 7 months and 12 years. The University of

Veterinary Medicine Budapest and the shelter cooperate in form of a castration program. During the lecture time every week from Monday to Thursday male and female shelter dogs are castrated by students under supervision of the responsible vets in the Small Animal Clinic for Obstetrics for education purpose.

For our study we were provided with the testicles of the male dogs from this castration program.

After the professional orchiectomy the testicles of one dog were collected in a sample container containing phosphate buffered saline. They were labelled with the associated identification code of the dog. The samples were immediately transferred to the laboratory for further process.

3.3 Sperm collection

Spermatozoa were collected for each dog from one testicle in fresh stadium and from the other testicle after cool storage at 4°C for 24 hours.

The steps for obtaining sperm from the cauda epididymis are the same for every sample and according to a defined concept.

Before sperm extraction, a sample container with 5ml of phosphate buffered saline + 20% fetal bovine serum (Sigma-Aldrich; Canada) must be prepared. Care must be taken here to ensure that all steps are carried out without contamination in order not to contaminate the samples. The sample container is labeled with the associated sample number and placed on a heating plate at 37 - 37.5°C.

To extract the sperm, the entire testicle is treated with a disinfectant at first, to prevent bacterial contamination of the sample.

After disinfection, the testicle is dried thoroughly and completely (*Figure 1*). The epididymis is then exposed using sterile cutlery. It is important to loosen the tough connective tissue layer, which is very strongly attached to the caput epididymis (*Figure 2*). Once this step is completed, the epididymal head is separated from the testicle (*Figure 3*). It is cut into small pieces and then placed in the prepared sample container (*Figure 4 and 5*), so that the sperm can flush out of the cauda epididymis. After this has been mixed for a few seconds using the Vortex, it is stored on the warming plate for 15 minutes so that the sperm have enough time to be able to flush out of the tissue.


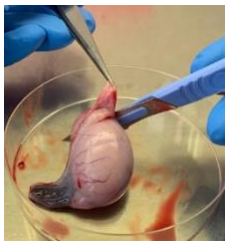
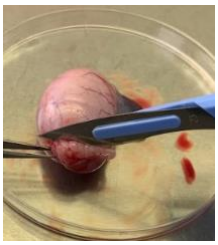
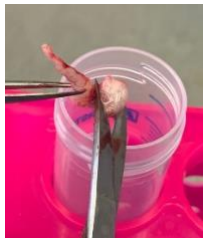


Figure 1 *Figure 2* *Figure 3* *Figure 4* *Figure 5*

Figure 1-5 **Epididymal sperm collection**

3.4 Equilibration

The equilibration is the preparation for the following freezing methods. During this process, the sperm is extended with the chosen extender and firstly cooled to 4°C.

Basically, equilibration is done to adjust the plasma membrane to low temperatures and to control the decrease of the metabolic activity of the spermatozoa [62] [63] [64].

In our study we were using the CaniPlus Freeze 1 Step extender (Minitüb GmBH, Germany). It consists of following components: purified water, sodium citrate, TRIS, glucose, glycerol, citric acid, proprietary factors and gentamicin.

At first, the extender has to be prepared. Therefore 20% of egg yolk has to be added to CaniPlus Freeze. In our case we needed only 0,5ml of prepared extender per sample, so 0,1ml of egg yolk was mixed with 0,4ml extender liquid.

In the next step 0,5ml of our sperm sample were filled into a sterile Eppendorf tube. Very slowly 0,5ml of CaniPlus Freeze was added to our sample. This happens drop by drop in a slightly horizontal position of the tube. The slow addition of the extender is very important in order to prevent the osmotically active substances from rushing into the cell. A result of this can be the diminishing of the cell membrane of the spermatozoa [62] [63] [64].

The final step is the immediate filling and sealing of the straws. In our experiment we needed 3 straws 0,25ml for the ultra-rapid freezing method. The remaining amount of the sample in the tube was used for the vitrification method. The straws and the Eppendorf tube were stored at 4°C for minimum 2 hours. As next step the cryopreservation with the 2 different protocols took place.

3.5 Ultra-rapid freezing

In order to prepare the ultra-rapid freezing method a styrofoam freezing box is provided with a freezing rack and liquid nitrogen. The rack is placed into the liquid nitrogen so that its surface is 4 - 5 cm above the liquid nitrogen level.

At this level the straws are placed into the vapor phase, where the temperature is -175°C. The correct placement is essential for the freezing process. The freezing time in this protocol is minimum of at least 20 minutes. After the 20 minutes incubation in the freezing box the straws are collected and submerged into the liquid nitrogen. They are finally put into a container filled with liquid nitrogen for preservation.

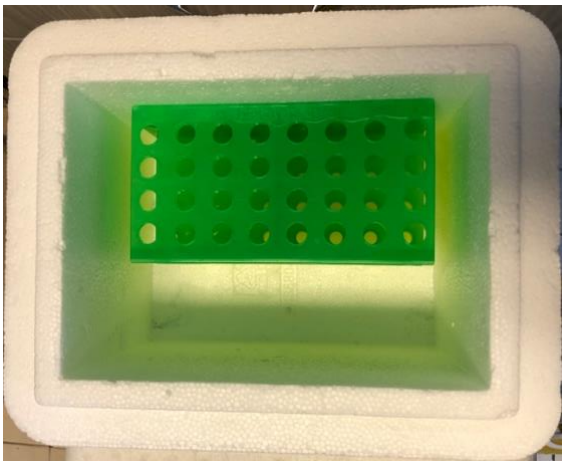

Figure 6 **Styrofoam freezing box for ultra-rapid freezing**

3.6 Vitrification

For the vitrification there are different claims regarding the freezing box. Since the freezing process occurs within seconds, the sample must be brought closer to the surface of the liquid nitrogen.

Therefore, the freezing box is filled with liquid nitrogen. As next step, a small bowl out of aluminum foil is placed right on the nitrogen surface, so that its bottom is in direct touch with the liquid phase. With the help of a Pasteur pipette the sample is dropped into the floating bowl. Frozen drops will be immediately formed out of the sample. Those drops are collected with a forceps and finally placed into a small sample tube. This tube is also stored in liquid nitrogen in a container for long time preservation.

Figure 7 **Freezing box for vitrification**

3.7 Semen examination

In the course of the semen examination the motility and the morphology analysis were performed.

3.7.1 Motility analysis

Regarding the motility analysis, the total and the progressive motility were evaluated by using the CASA system. It is important to carry out the motility analysis not later than 15 minutes after harvesting the spermatozoa. Examinations at a later point in time can result in the death of the cells.

Before the examination a slide is placed on the warming plate at 37 – 37,5°C. A pre-warmed slide is needed to ensure the optimal conditions during the analysis for the spermatozoa and to prevent the death of the cells.

After the sample is mixed again with help of a Pasteur pipette, 10 µl of the sample is placed on the pre-warmed slide and covered with a coverslip.

3.7.2 Morphology analysis

Regarding the morphology analysis, following parameters are evaluated: the normal morphology rate, the rate of acrosome defects, the rate of detached head cells and the rate of tail defects.

Before the microscopic analysis of the morphology of the spermatozoa can be performed, a stained smear must be prepared as first.

Therefore, 30µl of our sample is applied to one side of a prepared glass slide and then gently spread with help of another slide, to prevent the destruction of the cells.

The smear must be completely dry before further processing.

As next step, the dried smear must be stained. For this purpose, we use the Spermac staining kit (FertiPro, Belgium) (Fig. 3). It is consisting of a fixative and three staining solutions: stain A, the red stain, stain B, the pale green stain and stain C, the dark green stain.

First, our prepared smear is fixated for 15 minutes in a covered jar filled with the fixative. In the next step the slide is removed and placed vertically on an absorbent paper to drain excess fluid. It is important not to touch the specimen with the paper. The slide has to be completely dry before the next steps.

Afterwards, the slide is placed in stain A for 2 minutes. It is is then placed vertically on an absorbent paper to remove the excess stain and washed by gently dipping into a water jar for seven times. This washing process is repeated in a fresh water jar to ensure complete washing. After this, the excess water is drained off by touching the end of the slide onto absorbent paper.

The same steps are performed with stain B and stain C.

After the staining process, the slide is placed in vertical position on an absorbent paper for 5 to 10 minutes and then on the heating plate for 15 minutes to ensure complete drying.

Finally, the microscope-based morphology analysis can be started.

For this purpose, 100 sperm cells are counted in the stained smear at 100x magnification through the oil immersion objective. Their morphological characteristics and any changes are recorded in an Excel spreadsheet and subsequently evaluated.

In our study we focused on the following morphological abnormalities: acrosome defects, detached head cells and tail defects.

Figure 8 **Spermac stain**

3.7.3 proAKAP4 analysis

Assessment of proAKP4 concentration of spermatozoa was carried out following the manufacturer's protocol. A volume of 50 µL of thawed semen sample was mixed with 450 µL of the Bull Lysis Buffer and then processed for ELISA quantification using the Dog 4MID® Kit (4BioDx, Lille, France) according to the manufacturer's instructions. A quantity of 100 µL of lysates was put into each well of the anti-proAKAP4 antibody-coated plate. A secondary horseradish conjugated proAKAP4 antibody was then added to achieve the sandwich ELISA step. After suitable washing, the substrate solution was added, and coloration was stopped with the stop solution. Since color intensity is proportional to the amount of proAKAP4 present in each semen sample, optical density was measured by spectrophotometry at 450 nm. A standard curve was determined in parallel for precise concentrations of proAKAP4 in the bull semen samples. Results of proAKAP4 concentrations were always expressed in ng/ml. The final results are expressed in ng/10 millions of spermatozoa. According to the proAKAP4 concentration, samples can be categorized as follows:

ProAKAP4 concentration	**Semen Quality**	**Long lasting motility**
over 60 ng/ 10 million spermatozoa	**Excellent**	**+++**
60-40 ng/ 10 million spermatozoa	**Very good**	**++**
15-40 ng/ 10 million spermatozoa	**Good**	**+**
less than 15 ng/ 10 million spermatozoa	**Poor**	**+/-**

Table 1. Semen quality and morphology categories based on the proAKAP4 concentration, according to the manufacturer's protocol.

3.8 Statistical analysis

The data were analysed with R v3.0.0 software (R Development Core Team).

Difference of motility and morphology rate in controls and treated groups were analysed with One-Way Repeated Measures ANOVA (analysis of variance) test, with post hoc pairwise comparison using Tukey's Honest Significant Difference test.

Differences of excellent quality (based on proAKPA4 concentration) among groups was analysed with Fischer’s exact test. Differences at a probability value (p)<0.05 were considered significant.

4 Results

4.1 Motility

In regards of the motility analysis, in this study the total and the progressive motility were evaluated and all treatment groups were compared (***Figure 9*** *and* ***10***).

4.1.1 Total motility

Regarding the total motility no significant difference was found between the groups cryopreserved with ultra-rapid method compared to the fresh groups. However, we found significantly lower total motility in vitrified groups compared to both fresh and ultra-rapid freezing groups.

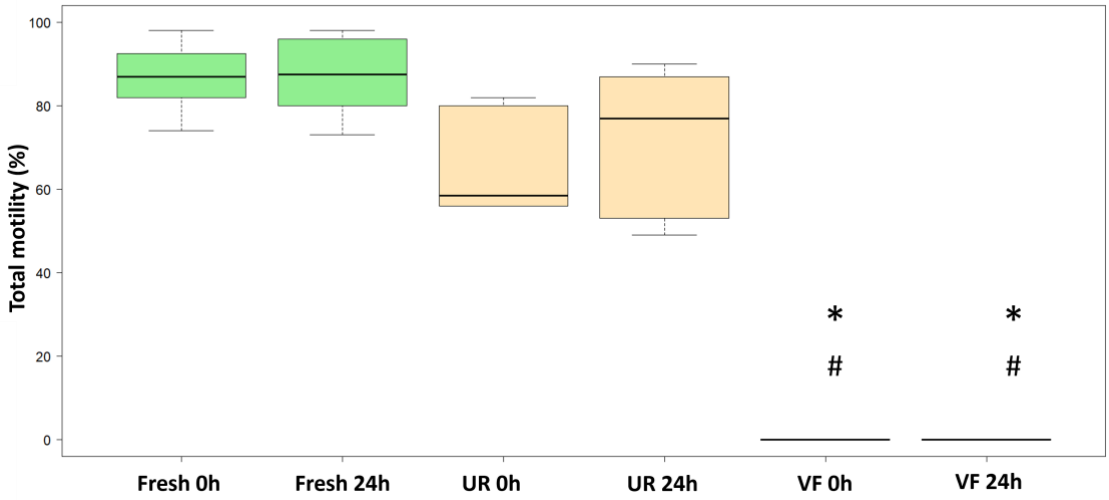

Figure 9 **Comparison of Total motility**

4.1.2 Progressive motility

There was no significant difference in the progressive motility between the UR 24h group compared to the Fresh 0h (p=0.47) and the Fresh 24h (p=0.17). However, we found significantly lower progressive motility in UR 0h group compared to both the Fresh 0h (p=0.007) and Fresh 24h groups (p=0.001). Progressive motility in groups of Vitrification (VF 0h and VF 24h) were also significantly lower than the Fresh groups (Fresh 0h and Fresh 24h).

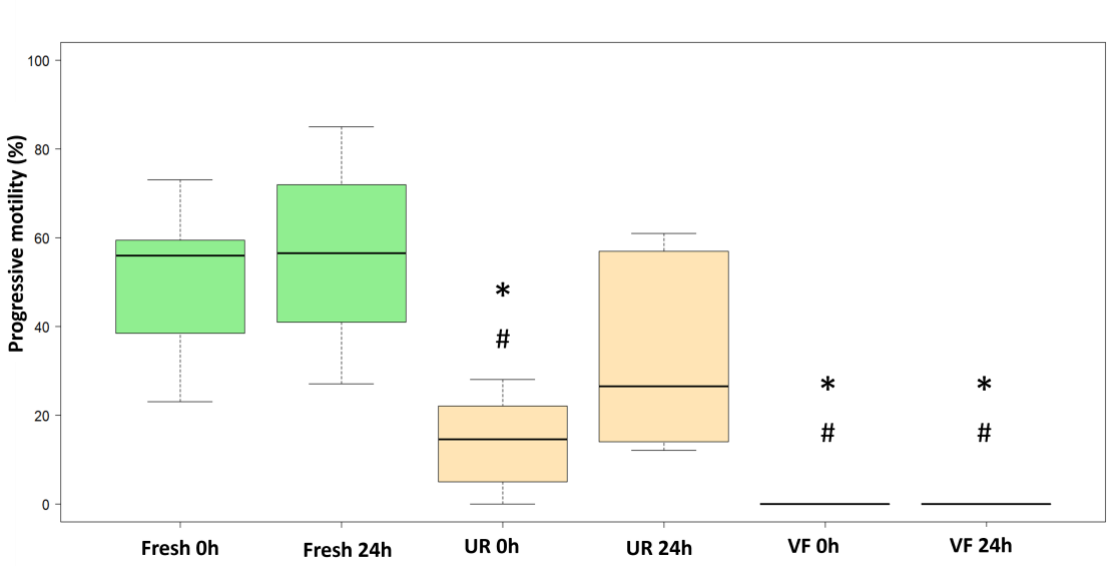

Figure 10 **Comparison of the Progressive motility**

4.2 Morphology

Regarding the morphology analysis, the normal morphology rate, the rate of acrosome defects, the rate of detached heads and the rate of tail defects were evaluated. The treatment groups were compared, as one can see in ***Figure 11-14***.

4.2.1 Normal morphology rate

There was no significantly difference between the 0h and 24h groups in any of the freezing methods. There was no effect of incubation ($p=0.1$) on the rate of cells with normal morphology. However, we found significant effect ($p<0.0001$) of freezing method. The rate of normal morphology cells was significantly lower in UR ($p<0.0001$) and also in VF ($p=0.0034$) compared to the Fresh samples.

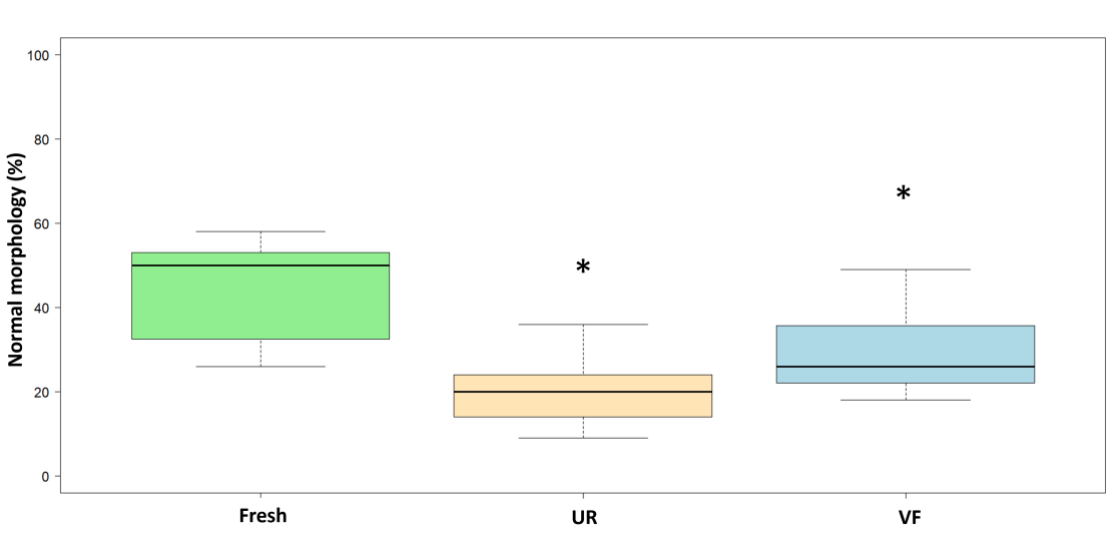

Figure 11 **Comparison of the Normal morphology rate**

4.2.2 Rate of acrosome defect

There was no significantly difference between the 0h and 24h groups in any of the freezing methods. There was no effect of incubation (p=0.2620) on the rate of cells acrosome defect. However, we found significant effect (p=0.0137) of the freezing method. The rate of the acrosome defect cells in UR (p =0.04) and in VF (p=0.01) was significantly higher compared to the fresh samples.

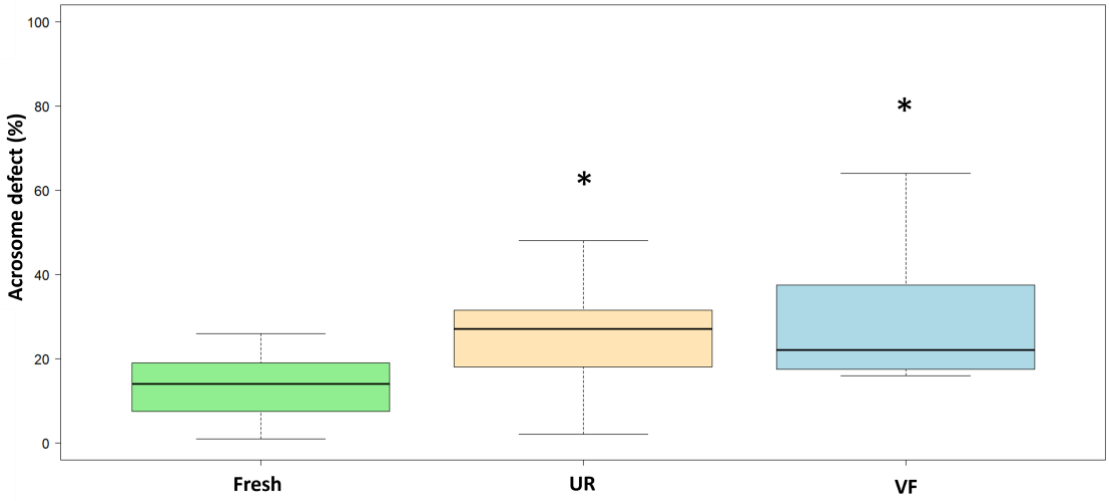

Figure 12 **Comparison of the Acrosome defect rate**

4.2.3 Rate of detached head cells

There was no significant effect of incubation (p=0.06) neither of cryopreservation method (p=0.52) on the rate of cells with detached head (***Figure 13***).

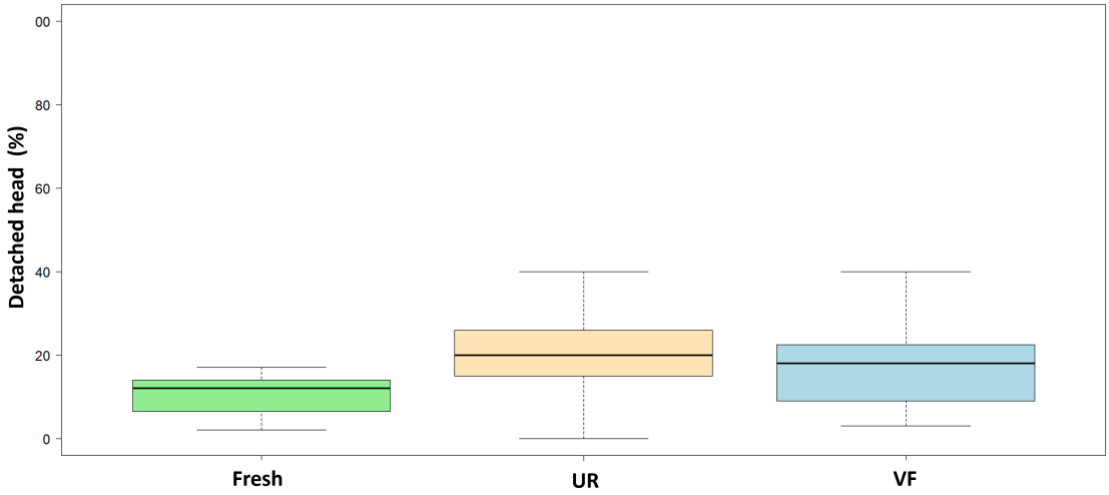

Figure 13 **Comparison of detached head cell rate**

4.2.4 Rate of tail defect

There was no significant effect of incubation (p=0.27) neither of the cryopreservation method (p=0.13) on the rate of cells with tail defect.

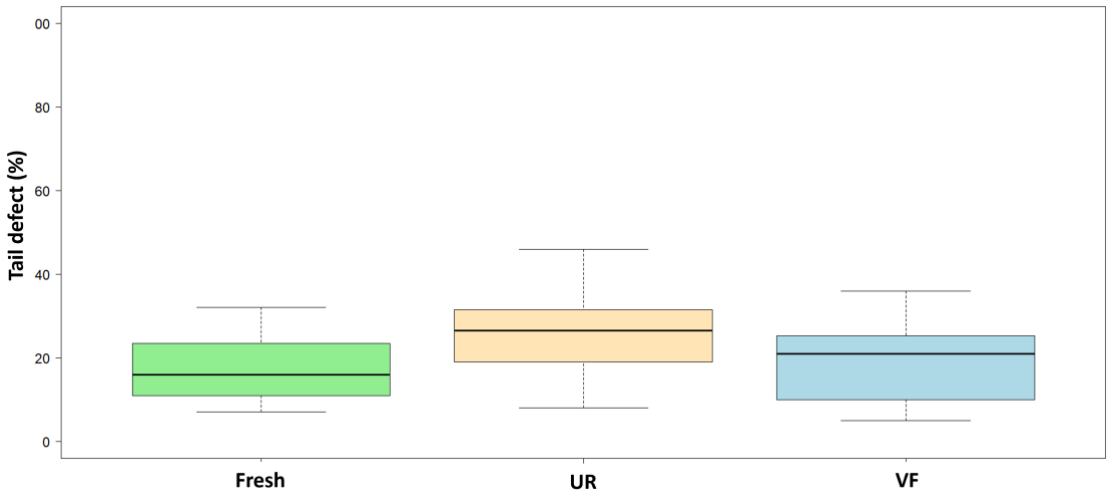

Figure 14 **Comparison of tail defect rate**

4.3 Pro AKAP4 concentration

	Semen quality			
Treatment	**Excellent**	**Very good**	**Good**	**Poor**
Fresh	**100 %** **(12/12)**	**-**	**-**	**-**
Ultra-rapid	**17%** **(2/12)**	**8.3%** **(1/12)**	**17%** **(2/12)**	**57.7%** **(7/12)**
Vitrification	**63.6%** **(7/11)**	**27.3%** **(3/11)**	**-**	**9.1%** **(1/11)**

Table 2. Results of the proAKPA4 concentration measurement in each treatment group.

Based on Fisher's exact test, rate of excellent quality samples was significantly lower in UR group ($p<0.0001$) and in Vitrification group ($p=0.03$) compared to Fresh ones. Furthermore, the rate in Vitrification was significantly higher compared to UR group ($p=0.03$).

5 Discussion

5.1 Motility

Regarding the comparison of the total motility, it can be said that the vitrification method produced the poorest results in our study.

By comparing the progressive motility, it becomes obvious that the outcome of the analysis of the vitrification method are again lower than the other groups.

Irrespective of the method of freezing, cryoinjuries are inevitable when it comes to preserving frozen semen. This is primarily attributed to the development of ice crystals both within and outside the sperm cells. The most noticeable consequence of cryopreservation is the reduction in sperm motility. This decline in motility is probably a result of heightened membrane permeability, which results in the outward diffusion of various molecules, including ATP. ATP is the essential energy source needed for sperm motility, but it becomes inaccessible to spermatozoa following exposure to cold shock [65].

In our study, the ultra-rapid freezing method causes lower cryoinjury than vitrification.

As a result of this, it is advised to use the ultra-rapid freezing method for semen cryopreservation to ensure a better total motility rate and thus better fertility.

5.2 Morphology

The comparison of the morphology analysis of the fresh, ultra-rapid and vitrified groups showed no significant differences in regards to the head and tail abnormalities.

Findings from the ultrastructural examination showed no variances in the morphological impairment of the membrane and acrosome among specimens subjected to either ultra-rapid freezing or vitrification [66]. However, in our study there was a significant higher rate of acrosome defect cells in ultra-rapid and vitrification compared to the fresh samples.

Cerdeira et al. discovered that wolf sperm acrosome had a greater sensitivity to vitrification-warming procedures. This might be explained by usage of inadequate sucrose concentrations for protection of the acrosome, among other factors [67].

It has been established that both dogs [68] [69] and red wolves [70] experience greater harm to their acrosomes when semen is frozen, as opposed to merely being stored at a cold temperature. When canine spermatozoa are cooled to 5 °C, there is only a modest

reduction in the percentage of spermatozoa with intact acrosomes. However, the freezing process inflicts significantly more damage [68].

5.3 ProAKAP4 concentration

In our study the proAKAP4 excellent rate was high in the fresh and vitrification groups. The ultra-rapid freezing groups achieved a poor proAKAP4 rate for the most part. Regarding the high excellent rate for the vitrification it is controversy, that the motility analysis provided opposite data in the vitrified samples. It is suspected that during the thawing process harmful effects or cryoinjury occurred, which can explain these controversy data. Further investigation is needed to clarify these findings. Another explanation can be provided by Le Couazer and Bencharif. They found out that even damaged spermatozoa can release proteins such as proAKAP4 and AKAP4 [61].

In stallions, there is a significant association between the concentration of proAKAP4 and both total and progressive motility in post-thawed semen [71]. In bulls, the concentration of proAKAP4 is linked to the degree of progressive motility in fresh semen [72]. In humans, the quantities of proAKAP4 and AKAP4 are associated with sperm motility [73] [74]. Notably, progressive motility plays a crucial role in the success of fertilization, especially in the context of in vitro fertilization [75] [76].

Similar correlations in regards to dogs haven’t been made until today.

It might be intriguing to investigate whether there is a potential link between proAKAP4 and fertility within this species, with the goal of enhancing the prospects of successful outcomes in both natural reproduction and artificial insemination procedures [61].

6 Abstract

Recent years have seen a drastic decline in biodiversity. In addition to the traditional in vitro conservation of genetic material, there is a need to establish a well-functioning protocol to cryopreserve the genetic material of a male of an endangered species or with valuable genetics. Storage of epididymal sperm is a feasible method to achieve this goal, however, time of sample delivery and freezing technique are key factors affecting the success rate.

Our aim was to investigate the freezing ability of canine epididymal spermatozoa in fresh stadium and after 24 hours storage at 4°C with two different freezing protocols (ultra-rapid freezing [UR] and vitrification [VF]).

The samples were collected from the tail of the epididymis from 10 mixed-breed dogs. Each sample (fresh and 24-hour storage) was cryopreserved by using the ultra-rapid and the vitrification method. Following parameters were evaluated immediately after harvesting, after 24 hours of cool storage of one testicle at 4°C and post-thawing: total and progressive motility, normal morphology rate, rate of acrosome defects, rate of detached heads, rate of tail defects and proAKAP4 concentration.

Concerning the total motility, no significant difference was found between the UR and fresh groups. There was no significant difference in the progressive motility between the UR 24h group compared to the fresh groups (Fresh 0h and Fresh 24h), but we found significantly lower progressive motility in the UR 0h group compared to both fresh 0h and 24h. However, significantly lower total and progressive motility in VF groups were found compared to both fresh and UR groups. Regarding the morphology analysis, incubation did not have an effect on any of the morphological parameters we examined in any of the freezing groups ($p=0.1$). In contrast, significant effect of freezing method ($p<0.0001$) on the rate of cells with normal morphology was found. The rate of normal morphology was significantly lower in the UR group and also in VF group compared to the fresh samples. A significant effect of the freezing method was also observed for acrosome defects ($p=0.0137$). The rate of sperms showing acrosome defect in UR ($p =0.04$) and in VF ($p=0.01$) was significantly higher compared to the fresh samples. In case of detached head and tail defects, in addition to incubation, the freezing method had no effect either. Assessing the proAKAP4 level, higher concentration was found in fresh and VF groups than that of UR group.

In our study, we found that total motility was not affected by incubation for 24 h, but the percentage of progressive motile cells was not significantly reduced in the group incubated for 24 h before freezing compared to fresh frozen samples. These data indicate that when transporting the heritable material of a freshly dead individual to the laboratory, it is recommended to store the epididymis at 4°C for 24 h to maintain better motility.

7 Összefoglalás

Az elmúlt években drasztikusan csökkenő biológiai sokféleség új kihívásokkal szembesít. A genetikai anyag hagyományos in vitro konzerválási módszerei mellett szükség van olyan protokollok kialakítására, amely alkalmas veszélyeztetett fajok hím egyedeinek, illetve genetikailag kiemelkedő hímek szaporító anyagának mélyhűtésére. Az epididimális spermiumok tárolása alkalmas módszer e cél elérésére, azonban a minta szállításának ideje és a fagyasztási technika kulcsfontosságú tényezők, amelyek befolyásolják a sikerességet.

Kutatásunk célja az volt, hogy megvizsgáljuk a kutya mellékhere spermiumok fagyasztási utáni minőségét izolálás után, illetve 24 órás 4°C-on történő tárolás után. A mélyhűtéshez két különböző módszert alkalmaztunk (ultrarapid mélyhűtés [UR] és vitrifikáció [VF]).

A spermiumokat keverék kanok (N=10) mellékheréinek farki részéből nyertük ki, kasztrálást követően azonnal, illetve 24 órás hűtve tárolást követően, majd UR és VF módszerekkel mélyhűtöttük. A vizsgált paraméterek a következők voltak: total (TM) és progresszív motilitás (PM), normál morfológiai arány, akroszóma hibák aránya, levált fejek aránya, farokhibák aránya és proAKAP4-koncentráció.

A TM tekintetében nem találtunk szignifikáns különbséget az UR és a friss csoport között. A PM tekintetében nem volt szignifikáns különbség az UR 24 órás csoport és a friss csoportok között (Friss 0 óra és Friss 24 óra), de az UR 0 órás csoportban szignifikánsan alacsonyabb PM-t mértünk, mint a friss 0 órás és 24 órás csoportban. A VF csoportokban szignifikánsan alacsonyabb TM és PM értékeket kaptunk mind a friss, mind az UR csoportokhoz képest. Ami a morfológiai elemzést illeti, a 24 órás hűtve tárolás egyik mélyhűtési módszer esetében sem volt hatással az általunk vizsgált morfológiai paraméterekre ($p=0,1$). Ezzel szemben a mélyhűtésnek szignifikáns hatása volt ($p<0,0001$) a normális morfológiájú sejtek arányára. A normál morfológiájú sejtek

aránya szignifikánsan alacsonyabb volt az UR csoportban és a VF csoportban is a friss mintákhoz képest. A mélyhűtés szignifikáns hatását figyeltük meg az akroszóma hibák esetében is (p=0,0137). Az UR (p=0,04) és a VF (p=0,01) csoportban az akroszóma hibát mutató spermiumok aránya szignifikánsan magasabb volt a friss mintákhoz képest. A levált fej és farok defektusok esetében a hűtve tárolás mellett a fagyasztási módszernek sem volt hatása. A proAKAP4 szintjét vizsgálva a friss és a VF csoportban magasabb koncentrációt találtunk, mint az UR csoportban.

Vizsgálatunk alapján elmondható, hogy a TM értéket nem befolyásolta a 24 órás inkubáció. A progresszív motilis sejtek aránya nem csökkent szignifikánsan a fagyasztás előtt 24 órán át inkubált csoportban a frissen fagyasztott mintákhoz képest. Ezek az adatok arra utalnak, hogy a frissen elhullott egyedek esetében ajánlott a herék (illetve velük együt a mellékherék) 24 órás, 4 °C-on történő tárolása a jobb motilitás megőrzése érdekében.

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