Submitted: 30 October, 2023 Accepted: 12 December, 2023 Published: 30 April, 2024 DOI:10.22514/jomh.2024.051

MINI-REVIEW

JOMH Journal of Men's Health

# Cryopreservation induces changes in the expression of noncoding RNAs in semen: a mini-review

Martin Štimpfel[1,2,*], Luka Bolha[3], Jože Pižem[3], Helena Ban-Frangež[1,2]

[1]Department of Human Reproduction, Division of Obstetrics and Gynaecology, University Medical Centre Ljubljana, 1000 Ljubljana, Slovenia
[2]Faculty of Medicine, University of Ljubljana, 1000 Ljubljana, Slovenia
[3]Institute of Pathology, Faculty of Medicine, University of Ljubljana, 1000 Ljubljana, Slovenia

***Correspondence**
martin.stimpfel@kclj.si
(Martin Štimpfel)

## Abstract

Semen cryopreservation is an important approach for preserving male fertility, and thawed semen is often used in *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) procedures. This process has been used for decades and seems both safe and efficient, despite causing cryodamage to spermatozoa, including the impairment of sperm functions, with decreased viability and motility being the main indicators of poor sperm quality post-thawing. Although an increase in sperm DNA fragmentation is common during cryopreservation, data on how cryopreservation affects sperm at the molecular level are scarce. There are especially limited data on the influence of sperm cryopreservation on spermatozoa non-coding RNA (ncRNA) stability and expression. To date, only three publications have explored this issue in human sperm samples, and there have been only a few more studies in animals, including mouse, bull, ram, boar and giant panda. The results of studies on human and animal semen indicate that cryopreservation affects ncRNA expression, which could crucially affect fertilization and embryonic development. Moreover, a study employing boar spermatozoa further showed that alterations in ncRNA expression also depend on the sperm cryopreservation protocol used, and a study employing human spermatozoa showed that microRNA (miRNA) expression changes during cryostorage. Therefore, the effects of cryopreservation on ncRNA expression in spermatozoa should be studied more thoroughly, mostly because sperm cryopreservation is important for medically assisted reproduction. Furthermore, despite the wide usage of sperm cryopreservation, how cryopreservation changes sperm at the molecular level and whether these changes have any effect on future generations have not been determined.

## Keywords

Sperm quality; Sperm cryopreservation; Sperm freezing; MicroRNAs; Non-coding RNAs

## 1. Introduction

Semen cryopreservation is an important approach for preserving male fertility. It is utilized primarily before gonadotoxic treatment in oncological patients with severe chronic medical conditions and in sperm donation, but it can also be used in severe oligozoospermia cases and ejaculatory disorder cases, and even to overcome logistic issues during *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) [1, 2]. Despite being performed for decades and proven safe and effective, the procedure may nevertheless have negative effect on post-thaw sperm quality, specifically decreased viability and motility, in clinical practice [3]. In addition to decreased viability and motility, which can easily be observed under a microscope immediately after thawing, impaired acrosome integrity [4] and increased sperm DNA fragmentation [4–6] can also be detected *via* additional analysis. When performing an *in vitro* fertilization with micromanipulation, for which just a few spermatozoa are sufficient, it is important to know that increased post-thaw sperm DNA fragmentation may lead to impaired embryo development and a lower pregnancy rate, even for sperm samples with normal conventional quality parameters [7]. Since the aforementioned cryodamage evidently has some detrimental effects on post-thaw sperm quality and the outcome of IVF/ICSI, studies have aimed to clarify how cryopreservation changes spermatozoa at the molecular level [8]. The main changes observed were related to the DNA methylation pattern, proteome and expression of coding RNAs (*i.e.*, mRNAs). While data on alterations in non-coding RNA (ncRNA) expression in spermatozoa following sperm cryopreservation are currently scarce, many studies have revealed an important role for sperm ncRNAs in embryonic development, the dysregulation of which can affect IVF/ICSI cycle outcomes [9–12]. For instance, the most often studied microRNA (miRNA) is miR-34c-5p, and an increase in its expression in semen was found to be correlated with a greater probability of obtaining viable embryos; however, when the level

of miR-449b-5p was increased, the probability of obtaining viable embryos was lower [13]. Furthermore, Yeh *et al*. [12] showed that the levels of miR-34b and miR-34c in semen were significantly higher in the group of patients who gave live birth. The importance of these miRNAs was confirmed in mice by injecting a miR-34c inhibitor into zygotes [14]. This intervention resulted in increased expression of miR-34c target mRNAs in early embryos, leading to differential expression of genes important for lipid metabolism, cellular membrane function, cell cycle phase transition, energy metabolism, vesicle organization, lipid biosynthetic process, and endomembrane system organization. Since cryopreservation has been shown to negatively affect miR-34c expression in human samples, which may have a detrimental effect on preimplantation embryo development [15], and since this is only one of the ncRNAs that may have an impact on preimplantation embryo development, it would be beneficial to obtain further insight into how cryopreservation affects ncRNA expression in sperm. Therefore, we focused this mini-review on data from human and animal studies on this topic.

## 2. Human cryopreserved semen and miRNA expression

Like most of the studies addressing the influence of cryopreservation on the epigenetic profile of spermatozoa, studies on ncRNAs have also been mostly conducted on animal models. To the best of our knowledge, there are currently only three studies in humans that specifically address alterations in miRNA expression due to cryopreservation and thawing [15–17]. Ezzati *et al*. [15] employed oligoasthenoteratozoospermic samples, which are especially delicate due to the low quality of the semen, and focused on the expression of two miRNAs (miR-34c and miR-184) along with two genes that interact with miR-34c/-184 and are involved in apoptosis (*P53* and *caspase 9*). The results of the study revealed that the expression levels of *P53*, miR-34c and miR-184 were decreased after thawing the semen sample, but the expression of *caspase 9* was increased. Furthermore, the levels of malondialdehyde (MDA) and superoxide dismutase (SOD) and the degree of sperm DNA fragmentation increased after thawing. Correlation analysis also revealed that miR-34c expression was negatively correlated with *P53* expression and immobility in samples from the severe oligoasthenoteratozoospermic group, while miR-184 expression was positively correlated with severe DNA fragmentation and immobility. A positive correlation between miR-34c expression and severe DNA fragmentation was observed in samples from the mild oligoasthenoteratozoospermic group. Expanding this study and additionally assessing the correlation between these parameters and altered miRNA expression levels after sperm thawing in other types of samples (*e.g.*, in normozoospermic samples or other cell types) would be interesting. The second study, performed by Xu *et al*. [16], revealed 21 differentially expressed miRNAs between frozen-thawed and fresh semen samples, with 18 miRNAs being significantly upregulated and three being downregulated in the frozen-thawed group. From the perspective of biological processes, these miRNAs were mostly involved in the extrinsic apoptotic signalling pathway in the absence of ligands, cellular response to DNA damage stimulus, actin cytoskeleton organization, *in utero* embryonic development, positive regulation of cell migration, and regulation of small nucleotide guanosine triphosphate hydrolase (GTPase)-mediated signal transduction. Although the main advantage of cryopreservation is the ability to store samples for an extended period of time, it would be beneficial to know if the alterations in the expression of molecules is related to the time of storage. Even short-term cryostorage alters miRNA expression in sperm. Seventy-eight miRNAs were found to be differentially expressed in the frozen-thawed samples compared to the fresh samples, and the number of differentially expressed miRNAs was increased in samples stored for longer periods (144 differentially expressed miRNAs after five years of storage, 187 miRNAs after 10 years, and 299 miRNAs after 15 years) [17]. There were 10 differentially expressed miRNAs that overlapped for all cryostorage times; eight (hsa-mir-509-3p, hsa-mir-514a-3p, hsa-mir-7154-5p, hsa-mir-67, hsa-mir-131, hsa-mir-506-3p, hsa-mir-143-3p and hsa-mir-27a-5p) were downregulated in the cryopreservation groups, and two were upregulated (hsa-mir-223 and hsa-mir-655-3p). The limitation of this study was the small number of included samples (only 3–4 for each cryostorage time). Nevertheless, the analysis revealed that half of the miRNA target genes whose expression was altered were expressed in oocytes, suggesting that additional research on this topic is needed since the use of such samples for fertilization could have a significant impact on reproductive outcomes.

## 3. Mouse cryopreserved semen and miRNA expression

While the mouse (*Mus musculus*) is probably the most commonly used animal model in medical research, data regarding the effect of semen cryopreservation on miRNA expression in mice are scarce. We could only find one such study that identified 19 upregulated and 76 downregulated miRNAs in frozen-thawed mouse semen compared to fresh semen [16]. From the perspective of biological processes, these miRNAs were mostly involved in the positive regulation of apoptosis, actin cytoskeleton organization, protein localization to the plasma membrane, and cell proliferation and development. An advantage of this study was that the authors compared the expression of differentially expressed miRNAs between fresh and frozen-thawed semen from mice and humans. They detected six homologous differentially expressed miRNAs (miR-148b-3p, miR-328-3p, miR-30b-5p, miR-106b-5p, miR-140-5p and miR-19b-3p) between humans and mice, which suggested that miRNA expression could be similarly influenced by cryopreservation across species and that, therefore, cryopreservation could have a similar effect on embryonic development across species [16]. This study further investigated the expression of three miRNAs (miR-148b-3p, miR-140-5p and miR-30b-5p) in mouse embryos, at different stages of embryonic development, when fresh mouse oocytes were fertilized by fresh or frozen-thawed semen. In all three cases, miRNA expression in embryos significantly differed at some point during development (day 1, day 2 or day 3.5). Notably, only the expression of miR-148b-3p was significantly lower

on all days in the frozen-thawed semen group, and the authors identified Pten (*PTEN*) as a target of miR-148b-3p, which showed increased expression in embryos at all stages.

## 4. Porcine cryopreserved semen and miRNA expression

To the best of our knowledge, the first published paper on the change in miRNA expression in sperm due to cryopreservation was authored by Zhang *et al*. [18] (2015), who attempted to identify a set of optimal endogenous reference control miRNAs for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis when studying cryopreservation of boar (*Sus scrofa*) spermatozoa. The authors studied 15 miRNAs (5S, let-7c-5p, ssc-mir-16-5p, ssc-mir-17-5p, ssc-mir-20a, ssc-mir-23a, ssc-mir-24-3p, ssc-mir-26a, ssc-mir-27a-3p, ssc-mir-92a, ssc-mir-103-3p, ssc-mir-106a, ssc-mir-107-3p, ssc-mir-186 and ssc-mir-221-3p), which were frequently used as endogenous controls in other studies, and determined that ssc-mir-186, ssc-mir-23a and ssc-mir-27a are probably the most stable and optimal choices for studying miRNA expression in their experimental setting, while ssc-mir-16-5p, ssc-mir-17-5p, and 5S were the least reliable. This conclusion was based on a comprehensive ranking considering mean rank values determined using the geNorm, NormFinder and BestKeeper algorithms. This study also revealed that some miRNAs could be differentially expressed after cryopreservation (let-7c-5p, ssc-mir-26a and ssc-mir-186), and it was later found that cryopreservation clearly changes miRNA expression in boar spermatozoa in a manner dependent on the protocol used for cryopreservation [19]. Therefore, we summarized all the cryopreservation methods used in the studies included in this review in Table 1 to highlight the similarities and differences between the most commonly used cryopreservation methods. Zhang *et al.* [19] tested the effects of two different approaches (cryopreservation with and without glycerol) for boar sperm cryopreservation, comparing the expression of 46 miRNAs between samples subjected to these two protocols and between frozen and fresh semen samples. Cryopreservation with glycerol resulted in the upregulation of 14 miRNAs when compared to fresh samples and in the upregulation of 17 miRNAs when compared to cryopreservation without glycerol. Conversely, only two miRNAs (miR-22 and miR-450b-5p) were downregulated in glycerol-treated samples compared to fresh semen samples, and only miR-24 was downregulated in samples cryopreserved without glycerol compared with fresh semen samples. The authors additionally showed that these differentially expressed miRNAs were associated with metabolic and cellular processes. The differentially expressed miRNAs identified in boar sperm after thawing by Dai *et al*. [20] were found to be significantly enriched in the Gene Ontology (GO) biological process terms “protein binding” and “response to stimuli”. High-throughput RNA sequencing (RNA-Seq) revealed that 135 miRNAs were differentially expressed between fresh and frozen-thawed boar semen. Notably, such an approach could provide crucial data regarding the processes that are most compromised by cryopreservation as well as valuable insights into how human semen cryopreservation protocols can be changed to minimize cryodamage. Moreover, it could be used to identify samples that might exhibit better recovery after thawing, especially in samples of very sensitive spermatozoa, as in the case of boar semen. For this reason, Pedrosa *et al*. [21] verified whether differences in miRNA expression correlated with changes in the quality of spermatozoa after thawing. They assessed miRNA expression in the extracellular vesicles of boar seminal plasma in addition to spermatozoa. They detected the overexpression of ssc-mir-503 in spermatozoa and the overexpression of ssc-mir-130a and ssc-mir-9 in extracellular vesicles from the seminal plasma in the low-viability sperm group compared to the high-viability sperm group. According to prediction analyses, these miRNAs are likely involved in the development of germ cells and in the production of energy and could even be used as biomarkers to predict the viability of boar semen samples after thawing.

The miRNA profile of frozen-thawed boar semen was also compared to that of giant panda (*Ailuropoda melanoleuca*) semen before and after cryopreservation [22]. The authors found that the number of differentially expressed miRNAs was lower in giant panda semen, but the number of differentially expressed target mRNAs was higher. GO analysis further revealed that most of the target mRNAs of the differentially expressed miRNAs in giant panda semen were associated with “membrane-associated” GO terms, while in boar, they were associated with “cell component-” and “cell process-associated” GO terms. Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of semen from both animals revealed that the targets of the differentially expressed miRNAs were mostly associated with “environmental information processing” and “organismal systems”, while the most enriched pathways were “olfactory transduction”, “pathways in cancer”, and “phosphoinositide 3-kinase /protein kinase B (PI3K-AKT) signaling pathway”. This study also assessed the difference in semen quality between the two animals after cryopreservation and determined that giant panda semen was more cryotolerant than boar semen, as the main quality parameters (*i.e.*, motility and viability) of giant panda semen were less impaired after thawing. In addition, the expression of long non-coding RNAs (lncRNAs) was also evaluated in giant panda semen [23], and high numbers of upregulated (1477) and downregulated (1396) lncRNA transcripts were detected between fresh and cryopreserved semen samples. The identified differentially expressed lncRNAs included 7689 predicted *cis* target genes and 1333 predicted *trans* target genes. GO analysis of the predicted *cis*-lncRNA targets revealed 202 significantly enriched terms, while 233 significantly enriched terms for the *trans*-lncRNA target genes. The significantly enriched GO terms for the predicted *cis*-lncRNA targets were sperm membrane-related terms, including “membrane”, “integral component of membrane”, and “plasma membrane”, while the five most enriched GO terms for the predicted *trans*-lncRNA targets were “cell development”, “Ral GTPase activator activity”, “metanephric loop of Henle development”, “metanephric distal tubule development” and “activation of Ral GTPase activity”. These data clearly reveal the impact of cryopreservation on spermatozoa at the molecular level, and a study by Ran *et al*. [23] also confirmed that lncRNAs and their target mRNAs were significantly differentially expressed in frozen-thawed

**TABLE 1. Summary of specified semen cryopreservation methods used in human and animal studies exploring noncoding RNA expression after semen cryopreservation.**

| Species | Cryopreservation protocol | Reference |
|---|---|---|
| Human | Quinn's advantage sperm freezing medium (SAGE media, Denmark) was used as the freezing medium. The samples were stored in 1.8 mL cryotubes. The cryotubes were cooled at 0.5 °C/min from 25 to −5 °C, held at −5 °C for 10 min, cooled at 10 °C/min rate from −5 to −80 °C and placed in liquid nitrogen (−196 °C). | [15] |
| Human | Sperm Freezing Medium (Origio, Denmark) was used as the freezing medium. The samples stored in cryotubes. The cryotubes were equilibrated at 37 °C for 10 min, incubated in liquid nitrogen vapour for 30 min and placed in liquid nitrogen | [16] |
| Human | The name/ingredients of the sperm freezing medium were not specified. The samples stored in 1.8 mL cryotubes. Cryopreservation was performed in a controlled-rate freezer. | [17] |
| Mouse | Cryopreservation was performed using R18S3 (Easycheck, Nanjing, China). The samples were stored in cryogenic vials. The vials were incubated for 10–15 min in liquid nitrogen vapour and then placed in liquid nitrogen. | [16] |
| Boar | Semen was washed with Beltsville thawing solution, and the pellet was cooled to 15 °C for 2 h. The pellet was resuspended in lactose-egg yolk extender, cooled at a rate of 0.2 °C per min to 4 °C and then diluted with glycerol to a final concentration of 3%. The samples were stored in 0.25 mL straws and frozen in a controlled-rate freezer. | [18] |
| Boar | Semen was washed with Beltsville thawing solution, and the pellet was cooled to 15 °C for 2 h. The pellet was resuspended in lactose-egg yolk extender, cooled in a refrigerator for 2 h to 4 °C and then diluted with glycerol to a final concentration of 3%. The samples were loaded into 0.25 mL straws and frozen in controlled-rate freezer. | [19] |
| Boar | Semen was diluted with Beltsville thawing solution and slowly cooled to 17 °C. After centrifugation, the pellet was resuspended in lactose-egg yolk extender, cooled to 4 °C and then diluted with glycerol to a final concentration of 3%. The samples were stored in 0.25 mL straws, equilibrated in liquid nitrogen vapour for 10 min and then placed in liquid nitrogen. | [20] |
| Boar | The samples were diluted in Beltsville Thawing Solution (BTS®-Minitube, Germany) and cooled at 17 °C for 24 h. After that, the samples were centrifuged, and the pellet resuspended in freezing extender Botu-Sui® fraction A (contains egg yolk) and cooled to 5 °C for 2 h. Then, freezing extender Botu-Sui® fraction B was added (contains 6% glycerol), and the mixture was loaded in a 0.5 mL straw. The samples were then equilibrated in liquid nitrogen vapour and placed in liquid nitrogen. | [21] |
| Giant panda | Semen samples were diluted with TEST egg yolk buffer (Irvine Scientific, USA) to a final concentration of 5% glycerol. The mixtures were loaded in 0.25 mL straws and cooled for 4 h to 4 °C. The straws were then placed 7.5 cm above liquid nitrogen for 1 min and 2.5 cm above liquid nitrogen for 1 min and then placed in liquid nitrogen. | [22, 23] |
| Bull | Semen samples were diluted in commercial extender (Bioxcell, IMV Technologies, France) and then loaded in 0.25-mL straws. The samples were cryopreserved by using a controlled-rate freezer utilizing the following programme: cooling from 4 to −10 °C at −5 °C/min; from −10 to −100 °C at −40 °C/min; and from −100 to −140 °C at −20 °C/min. | [24] |
| Bull | Semen was diluted with extender (main components: sodium citrate, egg yolk, fructose, glycerol, penicillin, streptomycin), cooled slowly from 25 °C to 4 °C and equilibrated for 4 h at 4 °C. Then, the samples were cooled to −140 °C within 8 min and placed in liquid nitrogen. | [25] |
| Bull | Semen was diluted with Triladyl®-egg yolk extender and loaded in 0.25 mL straws. The straws were then cooled to 4 °C for 24 h and then frozen in a control-rate freezer using the following programme: cooling at 5 °C/min to −10 °C, 40 °C/min to −110 °C and 20 °C/min to −140 °C. The straws were then placed in liquid nitrogen. | [26] |
| Ram | Semen sample were diluted with Tris + egg yolk extender at 38 °C, and the temperature was then lowered to 5 °C. After adding 5% glycerol, equilibration was performed for 3 h. The samples were loaded in 0.25 mL straws and frozen in a controlled-rate freezer. | [27] |

sperm compared to fresh semen, which indicates that these changes occur rapidly.

## 5. Bovine cryopreserved semen and miRNA expression

Another widely used animal for the analysis of sperm quality from various perspectives is the bull (*Bos taurus*) due to its notable agricultural importance. Fagerlind *et al*. [28] analysed miRNA expression in bovine semen and found many differentially expressed miRNAs whose expression correlated with fertility rates. In their study, the expression of 178 miRNAs was analysed, and the expression of seven miRNAs (miR-502-5p, miR-1249, miR-320a, miR-34c-3p, miR-19b-3p, miR-27a-5p and miR-148b-3p) was found to correlate with moderate and high nonreturn rates (*i.e.*, the proportion of cows that were not inseminated again during a specified period of time). Specifically, these miRNAs were significantly overexpressed in semen from bulls with moderate fertility. The authors concluded that alterations in miRNA expression likely resulted in negative regulation of crucial protein-coding genes in spermatozoa, leading to problems during pregnancy. In addition to the abovementioned miRNAs, miR-138 was shown to be expressed at significantly lower levels in the subfertile population (according to the nonreturn rate) than in the highly fertile population after thawing; furthermore, the expression of miR-138 was negatively correlated with sperm oxygen consumption [24]. Since cryopreserved sperm is predominantly used for cattle insemination, bovine sperm cryopreservation is important from the perspective of scientific research and has important commercial value. Therefore, several studies have been performed to identify miRNAs that are novel putative biomarkers of bovine sperm quality to aid the improvement of cryopreservation procedures and/or the identification of semen samples with the best possible quality. For instance, Shangguan *et al*. [25] revealed that 55 miRNAs and 526 mRNAs were differentially expressed in cryopreserved semen compared to fresh semen and that these miRNAs and mRNAs could influence fertility by affecting several biological processes, including fertilization, adenosine triphosphate (ATP)-related functions and apoptosis. Furthermore, Capra *et al*. [29] analysed high- and low-motility fractions of frozen-thawed bovine semen and identified 83 differentially expressed miRNAs and 79 putative Piwi-interacting RNAs (piRNAs). Almost half of these miRNAs (40) were related to apoptosis (increased apoptosis in the low motility sperm fraction), while changes in the expression of miR-17-5p, miR-26a-5p, miR-486-5p, miR-122-5p, miR-184 and miR-20a-5p mostly affected the PTEN, PI3K/AKT, and signal transducer and activator of transcription (STAT) signaling pathways. While these data suggest that semen cryopreservation affects spermatozoa miRNA expression in general, it has also been revealed that miRNA expression profiles differ among samples of sex-sorted bovine sperm prior to cryopreservation [26]. Although sperm sex sorting is not ethically acceptable in assisted human reproduction, it is allowed and is of great commercial interest in cattle. For this reason, Keles *et al*. [26] analysed miRNA expression in bovine semen samples from high- and low-fertility bulls by employing conventionally cryopreserved semen and sex-sorted semen. Using RNA-Seq, they detected 85 miRNAs and found that only miR-10a-5p and miR-9-5p were differentially expressed between high- and low-fertility bulls. Interestingly, the expression levels of miR-9-5p, miR-34c, miR-423-5p, miR-449a, miR-5193-5p, miR-1246, miR-2483-5p, miR-92a and miR-21-5p were significantly correlated with the nonreturn rate when sex-sorted semen was used for insemination, but they were not correlated with the nonreturn rate when conventionally cryopreserved bovine semen was used.

## 6. Ovine cryopreserved semen and miRNA expression

Like cattle, sheep (*Ovis aries*) are regarded as important agricultural animals in several countries; therefore, obtaining the best quality ram semen after cryopreservation is highly desirable. Nonetheless, studies on the effect of cryopreservation on ncRNA dysregulation are scarce. The following differences in miRNA expression after freezing-thawing of ram semen were identified. Compared to those in control samples, the expression of let-7a, miR-485, and miR-29a was significantly downregulated in cryopreserved semen, while the expression of miR-127 was significantly upregulated [27]. The authors proposed that these molecular alterations could cause motility and morphological disorders in ram semen following sperm freeze-thawing since the determined molecular changes are mostly associated with lipid peroxidation in spermatozoa.

## 7. Conclusion

The data presented in this review suggest that cryopreservation affects ncRNA expression (the data about dysregulated ncRNAs after semen cryopreservation reported in all included studies are summarized in Table 2) and that aberrantly expressed ncRNAs could affect fertilization and embryonic development. Notably, the expression of ncRNAs might also be influenced by the sperm cryopreservation protocol used, but there are no data on whether freezing media from different manufacturers, despite being similar in composition, have any impact on sperm quality. Although many semen samples are cryostored for many years or even decades, the negative impact of prolonged cryostorage on ncRNA expression represents a major drawback in promoting semen cryostorage. Therefore, further studies on this topic are of utmost importance, primarily because human sperm cryopreservation is widely used in clinical practice, and there are still many uncertainties regarding how this procedure changes sperm at the molecular level and whether these changes have any effect on future generations.

**TABLE 2. Dysregulated ncRNAs in semen after cryopreservation.**

| Species | Number of detected ncRNAs[a] | Number of dysregulated ncRNAs[b] | Validated ncRNAs[c] | Expression[d] | Reference |
|---|---|---|---|---|---|
| Human | NA | 2 miRNAs | hsa-mir-34c, hsa-mir-184 | ↓ | [15] |
| Human | NA | 21 miRNAs (18 ↑, 3 ↓) | hsa-mir-140-5p, hsa-mir-19b-3p, hsa-mir-664a-3p, hsa-mir-509-3-5p, hsa-mir-106b-5p, hsa-mir-30a-5p, hsa-mir-342-3p | ↑ | [16] |
| | | | hsa-mir-328-3p, hsa-mir-590-3p, hsa-mir-210-5p | ↓ | |
| Mouse (*M. musculus*) | NA | 95 miRNAs (19 ↑, 76 ↓) | mmu-mir-140-5p | ↑ | [16] |
| | | | mmu-mir-148b-3p, mmu-mir-30b-5p | ↓ | |
| Human | 650–750 miRNAs | 78–299 miRNAs | hsa-mir-143-3p, hsa-mir-506-3p, hsa-mir-514a-3p, hsa-let-7d-3p, hsa-let-7i-3p | ↑ | [17] |
| | | | hsa-mir-655-3p, hsa-let-7c-5p | ↓ | |
| Boar (*S. scrofa*) | NA | 15 miRNAs | let-7c-5p, ssc-mir-26a, ssc-mir-186 | ↓ | [18] |
| | | | ssc-mir-16-5p, ssc-mir-17-5p, ssc-mir-20a, ssc-mir-23a, ssc-mir-24-3p, ssc-mir-27a-3p, ssc-mir-92a, ssc-mir-103-3p, ssc-mir-106a, ssc-mir-107-3p, ssc-mir-221-3p, 5S | NS | |
| Boar (*S. scrofa*) | NA | 46 miRNAs Cryopreservation without glycerol (7 ↓, 2 ↑) Cryopreservation with 3% glycerol (14 ↑, 2 ↓) | let-7a, let-7c, let-7d, let-7e, let-7f-5p, let-7i, ssc-mir-9-5p, ssc-mir-26a, ssc-mir-98, ssc-mir-181a, ssc-mir-186, ssc-mir-212, ssc-mir-374a-5p, ssc-mir-374b-5p | ↑ | [19] |
| | | | ssc-mir-22, ssc-mir-34c, ssc-mir-124, ssc-mir-181a, ssc-mir-186, ssc-mir-224, ssc-mir-450b-5p, ssc-mir-450c-5p | ↓ | |

**TABLE 2. Continued.**

| Species | Number of detected ncRNAs[a] | Number of dysregulated ncRNAs[b] | Validated ncRNAs[c] | Expression[d] | Reference |
|---|---|---|---|---|---|
| Boar (*S. scrofa*) | Fresh sperm: 1028 miRNAs Frozen-thawed sperm: 984 miRNAs | 135 miRNAs (101 ↑, 34 ↓) | ssc-mir-126, ssc-mir-212, ssc-mir-128, unconservative_X_272462, conservative_15_100875 | ↓ | [20] |
| | | | ssc-mir-186 | ↑ | |
| Boar (*S. scrofa*) | NA | 258 miRNAs in spermatozoa 54 miRNAs in EVs from seminal plasma | ssc-mir-9, ssc-mir-130a, ssc-mir-503 | ↑ | [21] |
| Giant panda (*A. melanoleuca*) | 899 miRNAs | 284 miRNAs (195 ↑, 89 ↓) | unconservative_NW_003217675.1_185862, unconservative_NW_003220474.1_435144, conservative_NW_003217342.1_51663, conservative_NW_003217890.1_245248, conservative_NW_003218322.1_322741, conservative_NW_003219491.1_415457, conservative_NW_003219117.1_397928, conservative_NW_003217617.1_168051, conservative_NW_003218322.1_322742 | ↓ | [22] |
| Giant panda (*A. melanoleuca*) | 22774 lncRNAs | 2873 lncRNAs (1477 ↑, 1396 ↓) | MSTRG.436078.1, MSTRG.436078.2, MSTRG.516446.1 | ↑ | [23] |
| Bull (*B. taurus*) | 178 miRNAs | 95 miRNAs | NA | NA | [28] |
| Bull (*B. taurus*) | NA | 8 out of 10 included miRNAs | bta-mir-138 | ↓ | [24] |
| | | | bta-mir-7, bta-mir-10b, bta-mir-19b, bta-mir-26a, bta-mir-146b, bta-mir-449a, bta-mir-495 | NS | |
| | | | bta-mir-10a, bta-mir-34a | NA | |

**TABLE 2. Continued.**

| Species | Number of detected ncRNAs[a] | Number of dysregulated ncRNAs[b] | Validated ncRNAs[c] | Expression[d] | Reference |
|---|---|---|---|---|---|
| Bull (*B. taurus*) | 210 miRNAs | 55 miRNAs (31 ↑, 24 ↓) | NA | NA | [25] |
| Bull (*B. taurus*) | 813 miRNAs | 83 miRNAs | NA | NA | [29] |
| | 99 putative piRNA clusters in HM<br>51 putative piRNA clusters in LM | 79 putative piRNA clusters | NA | NA | |
| Bull (*B. taurus*) | 85 miRNAs | NA | NA | NA | [26] |
| Ram (*O. aries*) | NA | 31 out of 37 included miRNAs | miR-107, miR-127 | ↑ | [27] |
| | | | miR-29a, miR-485, let-7a | ↓ | |
| | | | miR-99a, miR-21, miR-376a-5p, miR-125b, miR-25, miR-22-3p, miR-10a, miR-10b, miR-369-5p, miR-16, miR-200a, miR-23b, miR-409-3p, miR-30a, miR-152, miR-323-3p, miR-148a, miR-374, miR-27a, miR-3958-3p, miR-494, miR-410, miR-150, mir-19b, miR-191, miR-181a | NS | |
| | | | miR-143, miR-199, miR-379-5p, miR-411b-3p, miR-432, miR-1197 | NA | |

*ncRNA, non-coding RNA; NA, not applicable; NS, not significant; EV, extracellular vesicle; HM, high motility sperm fraction; LM, low motility sperm fraction; ↓, downregulation; ↑, upregulation.*

[a] *Total number of detected ncRNAs.*

[b] *Total number of studied ncRNAs and/or total number of identified differentially expressed ncRNAs in cryopreserved semen compared to control semen.*

[c] *ncRNAs whose expression was validated by qPCR.*

[d] *Expression of validated ncRNAs.*

## AVAILABILITY OF DATA AND MATERIALS

Data sharing is not applicable to this article, as no datasets were generated or analysed during the current study.

## AUTHOR CONTRIBUTIONS

MŠ and LB—conception and design of the study; literature review. MŠ—manuscript drafting. LB, JP and HBF—critical revision of the manuscript. All the authors revised and edited the important contents of the manuscript and read and approved the final version.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## ACKNOWLEDGMENT

Not applicable.

## FUNDING

This research was funded by the Slovenian Research Agency (project no. J3-2531) and UMC Ljubljana, Slovenia (tertiary project no. 20210024).

## CONFLICT OF INTEREST

The authors declare no conflict of interest. Martin Stimpfel is serving as one of the Editorial Board members of this journal. We declare that Martin Stimpfel had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to AW.

## REFERENCES

[1] Grin L, Girsh E, Harlev A. Male fertility preservation—methods, indications and challenges. Andrologia. 2021; 53: e13635.

[2] Borate GM, Meshram A. Cryopreservation of sperm: a review. Cureus. 2022; 14: e31402.

[3] Ozimic S, Ban-Frangez H, Stimpfel M. Sperm cryopreservation today: approaches, efficiency, and pitfalls. Current Issues in Molecular Biology. 2023; 45: 4716–4734.

[4] Gómez-Torres MJ, Medrano L, Romero A, Fernández-Colom PJ, Aizpurúa J. Effectiveness of human spermatozoa biomarkers as indicators of structural damage during cryopreservation. Cryobiology. 2017; 78: 90–94.

[5] Le MT, Nguyen TTT, Nguyen TT, Nguyen TV, Nguyen TAT, Nguyen QHV, *et al*. Does conventional freezing affect sperm DNA fragmentation? Clinical and Experimental Reproductive Medicine. 2019; 46: 67–75.

[6] Gouhier C, Pons-Rejraji H, Dollet S, Chaput L, Bourgne C, Berger M, *et al*. Freezing does not alter sperm telomere length despite increasing DNA oxidation and fragmentation. Genes. 2023; 14: 1039.

[7] Du C, Tuo Y. Correlation of DNA fragments with routine semen parameters and lifestyle and their impact on assisted reproductive outcomes. Revista Internacional De Andrología. 2023; 21: 100337.

[8] Ďuračka M, Benko F, Tvrdá E. Molecular markers: a new paradigm in the prediction of sperm freezability. International Journal of Molecular Sciences. 2023; 24: 3379.

[9] Tomic M, Bolha L, Pizem J, Ban-Frangez H, Vrtacnik-Bokal E, Stimpfel M. Association between sperm morphology and altered sperm microRNA expression. Biology. 2022; 11: 1671.

[10] Vallet-Buisan M, Mecca R, Jones C, Coward K, Yeste M. Contribution of semen to early embryo development: fertilization and beyond. Human Reproduction Update. 2023; 29: 395–433.

[11] Li H, Li L, Lin C, Hu M, Liu X, Wang L, *et al*. Decreased miR-149 expression in sperm is correlated with the quality of early embryonic development in conventional *in vitro* fertilization. Reproductive Toxicology. 2021; 101: 28–32.

[12] Yeh LY, Lee RK, Lin MH, Huang CH, Li SH. Correlation between sperm micro ribonucleic Acid-34b and -34c levels and clinical outcomes of intracytoplasmic sperm injection in men with male factor infertility. International Journal of Molecular Sciences. 2022; 23: 12381.

[13] Conflitti AC, Cicolani G, Buonacquisto A, Pallotti F, Faja F, Bianchini S, *et al*. Sperm DNA fragmentation and sperm-borne miRNAs: molecular biomarkers of embryo development? International Journal of Molecular Sciences. 2023; 24: 1007.

[14] Cui L, Fang L, Zhuang L, Shi B, Lin CP, Ye Y. Sperm-borne microRNA-34c regulates maternal mRNA degradation and preimplantation embryonic development in mice. Reproductive Biology and Endocrinology. 2023; 21: 40.

[15] Ezzati M, Shanehbandi D, Bahramzadeh B, Hamdi K, Pashaiasl M. Investigation of molecular cryopreservation, fertility potential and microRNA-mediated apoptosis in oligoasthenoteratozoospermia men. Cell and Tissue Banking. 2021; 22: 123–135.

[16] Xu X, Li W, Zhang L, Ji Y, Qin J, Wang L, *et al*. Effect of sperm cryopreservation on miRNA expression and early embryonic development. Frontiers in Cell and Developmental Biology. 2021; 9: 749486.

[17] Huang C, Ji XR, Huang ZH, Liu Q, Wang RJ, Fan LQ, *et al*. Long-term storage modifies the microRNA expression profile of cryopreserved human semen. To be published in Biomolecules & Biomedicine. 2023. [Preprint].

[18] Zhang Y, Zeng C, He L, Ding L, Tang K, Peng W. Selection of endogenous reference microRNA genes for quantitative reverse transcription polymerase chain reaction studies of boar spermatozoa cryopreservation. Theriogenology. 2015; 83: 634–641.

[19] Zhang Y, Dai D, Chang Y, Li Y, Zhang M, Zhou G, *et al*. Cryopreservation of boar sperm induces differential microRNAs expression. Cryobiology. 2017; 76: 24–33.

[20] Dai DH, Qazi IH, Ran MX, Liang K, Zhang Y, Zhang M, *et al*. Exploration of miRNA and mRNA profiles in fresh and frozen-thawed boar sperm by transcriptome and small RNA sequencing. International Journal of Molecular Sciences. 2019; 20: 802.

[21] Pedrosa AC, Andrade Torres M, Vilela Alkmin D, Pinzon JEP, Kitamura Martins SMM, Coelho da Silveira J, *et al*. Spermatozoa and seminal plasma small extracellular vesicles miRNAs as biomarkers of boar semen cryotolerance. Theriogenology. 2021; 174: 60–72.

[22] Ran MX, Zhou YM, Liang K, Wang WC, Zhang Y, Zhang M, *et al*. Comparative analysis of MicroRNA and mRNA profiles of sperm with different freeze tolerance capacities in Boar (*Susscrofa*) and Giant Panda (*Ailuropodamelanoleuca*). Biomolecules. 2019; 9: 432.

[23] Ran MX, Li Y, Zhang Y, Liang K, Ren YN, Zhang M, *et al*. Transcriptome sequencing reveals the differentially expressed lncRNAs and mRNAs involved in cryoinjuries in frozen-thawed Giant Panda (*Ailuropoda melanoleuca*) Sperm. International Journal of Molecular Sciences. 2018; 19: 3066.

[24] Salas-Huetos A, Ribas-Maynou J, Mateo-Otero Y, Tamargo C, Llavanera M, Yeste M. Expression of miR-138 in cryopreserved bovine sperm is related to their fertility potential. Journal of Animal Science and Biotechnology. 2023; 14: 129.

[25] Shangguan A, Zhou H, Sun W, Ding R, Li X, Liu J, *et al*. Cryopreservation induces alterations of miRNA and mRNA fragment profiles of bull sperm. Frontiers in Genetics. 2020; 11: 419.

[26] Keles E, Malama E, Bozukova S, Siuda M, Wyck S, Witschi U, *et al*. The micro-RNA content of unsorted cryopreserved bovine sperm and its relation to the fertility of sperm after sex-sorting. BMC Genomics. 2021; 22: 30.

[27] Güngör BH, Tektemur A, Arkali G, Dayan Cinkara S, Acisu TC, Koca RH, *et al*. Effect of freeze-thawing process on lipid peroxidation, miRNAs, ion channels, apoptosis and global DNA methylation in ram spermatozoa. Reproduction, Fertility, and Development. 2021; 33: 747–759.

[28] Fagerlind M, Stålhammar H, Olsson B, Klinga-Levan K. Expression of miRNAs in bull spermatozoa correlates with fertility rates. Reproduction in Domestic Animals. 2015; 50: 587–594.

[29] Capra E, Turri F, Lazzari B, Cremonesi P, Gliozzi TM, Fojadelli I, *et al*. Small RNA sequencing of cryopreserved semen from single bull revealed altered miRNAs and piRNAs expression between high- and low-motile sperm populations. BMC Genomics. 2017; 18: 14.

**How to cite this article:** Martin Štimpfel, Luka Bolha, Jože Pižem, Helena Ban-Frangež. Cryopreservation induces changes in the expression of noncoding RNAs in semen: a mini-review. Journal of Men's Health. 2024; 20(4): 19-28. doi: 10.22514/jomh.2024.051.