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HYPOTHERMIC STORAGE OF STURGEON SPERM: METHODOLOGY AND ONGOING HISTORY

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Abstract. *Hypothermic storage of sperm in liquid state without freezing, without the use of either liquid nitrogen or dry ice as well as special cryological equipment, is an interesting and attractive research line in reproductive biology in terms of practical application. Historically, hypothermia is the very first approach to preservation of genetic material, but, despite this, the methods of hypothermic storage of gametes and embryos have not received proper development and application in animal husbandry, giving way to cryopreservation. One of the main reasons for this is high species-specific resistance to cold storage. The technologies for hypothermic storage of sperm existing today and recommended for use in fish farming and in sturgeon breeding in particular are still not effective enough and require further improvement. This short review outlines the history of the development of technologies for the hypothermic storage of sturgeon sperm, and considers a number of methodological approaches, concepts and ideas behind these developments. The male reproductive system in sturgeons, the structure and physiology of spermatozoa have a number of features that distance them from teleost fishes, but partly relate to amphibians and higher vertebrates. This made some successful approaches and achievements in the development of methods for hypothermic storage of mammalian (mouse and human) sperm possible to apply to sturgeons. Thus, the most effective and possibly promising approach is partial or complete replacement of seminal plasma with salt-free isotonic solutions based on sugars (oligosaccharides) and albumin. The purpose of this review is to draw the attention of fish farmers and researchers to developments and advances in hypothermic sperm storage.*

Key words: *sturgeons, sperm storage, hypothermia.*

The most natural way to preserve biological objects and products of biological origin is cooling or even complete freezing, which leads to slowing chemical and biochemical reactions. Preservation and restoration of the viability of biological objects (cells, tissues or whole organisms) after exposure to low temperatures is achieved due to a reversible decrease or cessation of metabolism, which is known as low-temperature anabiosis (suspended animation). This physiological state is often referred to as 'hypothermia'. The need to preserve the sperm of farm animals for a certain period is associated with the emergence and development of artificial insemination technologies, since, first, it allows postponing artificial insemination and thus flexibly planning reproductive procedures, and, second, makes it possible to transport the genetic material. The first is especially important in fish breeding, as it allows

solving the problem of non-simultaneous maturation of gametes in males and females during spawning campaigns.

A brief historical background of hypothermic preservation of sperm in vertebrates

The documented history of scientific research on hypothermic storage of sperm begins with the experiments carried out at the end of the 18th century by the Italian abbot Lazzaro Spallanzani, who cooled stallion, bovine and human semen in snow [36]. Studying the nature and properties of animal and human sperm, Spallanzani performed numerous experiments, including effects of low temperature on sperm motility. In his famous "Tracts on the natural history of animals and vegetables" (English edition 1803), Spallanzani described an experiment with cooling stallion semen in the snow: *"Observing that the vermiculi had become motionless, I took the glass from the snow, and left it exposed to the air at 81°* (degrees Fahrenheit; equals to +27.2 °C – author's note). *An hour later, it astonished me to find all the vermiculi* (spermatozoa – author's note) *reanimated in such a manner as if they had just come from the seminal vessels"*. Spallanzani obtained a similar result by cooling the semen of a bull and a human for 5—10 min in the snow.

Spallanzani's experiments became the first scientific basis for the hypothermic preservation of animal sperm. The development and widespread use of artificial insemination technologies in animals in the late 19th – early 20th centuries, mainly thanks to the works of the Russian scientist Ilya Ivanov [21], required the improvement of sperm preservation techniques to prolong enough the term of storage to transport or to plane inseminations. By the 40s, egg yolk-based buffer extenders were developed in which the semen of farm animals can be stored at temperatures of about + 5 °C for at least 3 days [25, 33].

In aquaculture, the ability of fish sperm to be stored for a short time at temperatures from about zero to + 5 °C has been known for a long time [9]. The first experimental developments for the milt of salmonids were carried out as early as in 1940-s [34]. The work of Butcher (1944) is considered the first published successful experience of preserving the milt of teleost fish without freezing for more than a day: the semen of the river trout *Salmo fario* maintained the ability to fertilize for 5 days, keeping under paraffin oil at temperatures of +2.2...+3.3 °C [17]. T.A. Detlaf et al. [35] pointed to earlier works by A.I. Shmidtov (1936) [13] and G.M. Persov (1941) [7], where sturgeon milt was stored on ice for 5–6 days.

In 1949 Polge et al. reported successful cryopreservation of human sperm at –79 °C using glycerol as a cryoprotective agent (cryoprotectant) [27]. This work is believed to initiate extensive basic research in cryobiology and the development of practical approaches to cryopreservation of biological objects. Industrial mass production of liquid nitrogen after World War II made the idea of long-term storage of biological objects at ultra-low temperatures very attractive and thus contributed to the advancement of cryobiology. Cryopreservation has become the mainstream in the storage of animal and human sperm, allowing creation of cryobanks and genetic collections to store genetic material indefinitely and transport over long distances. In the early 1950s many works were published on the cryopreservation of sperm in various animal species, including fishes [16].

At the same time, the importance of the hypothermic storage of sperm without freezing had not diminished, because it could adequately solve problems that are not related to long-term storage of male gametes. Considerably

losing out to cryopreservation in terms of duration, hypothermic storage has a number of advantages: it does not depend on sources of liquid nitrogen or dry ice, does not require special cryologic equipment, and makes transportation of genetic material safe and convenient. In addition, the development of methods for cryopreservation of sperm in various animals from the very beginning faced many challenges, including either species specificity or dependence on the initial quality of each sperm batch, which complicates the standardization of technologies during implementation and reduces efficiency and reproducibility of results. Thus, along with the development of methods for cryopreservation of sperm, the search for effective approaches to hypothermic storage is currently ongoing.

Basic concepts in the methodology for hypothermic storage of fish sperm

There are three interfering concepts that stand out clearly in the development of methods and technologies for preserving animal semen without freezing:

Physiological sperm preservation. The concept is based on the well-known phenomenon of long-term preservation of sperm in many animals in an inactive state in the genital tract or specialized organs associated with it, both in males and females [11]. It is noteworthy that sperm is preserved at the temperatures of the metabolic activity of the cells. In light of this concept, fishes are interesting in that before entering the water mature spermatozoa in the gonads are in an inactive dormant state.

Chemical anabiosis (chemobiosis). This concept consists in the reversible suppression of metabolism and destructive chemical and physico-chemical processes using certain chemicals [12].

Hypothermic storage. Cooldown leads to a decrease in both enzymatic activity and the rate of chemical reactions in general. An important remark should be made here regarding the applicability of the concept of 'hypothermia' to fishes, which are poikilothermic (ectothermic) organisms and for many of them near-zero positive temperatures are physiologically normal. So, for the spermatozoa of these fish it is more correct to discuss the state of hypothermic hypobiosis maintained due to special physico-chemical and/or physiological and biochemical conditions. This makes it impossible to develop technologies for hypothermic storage of fish sperm strictly within the framework of the concept of hypothermia only and requires knowledge of physiology and biochemistry of sperm for each species.

Development of technologies for hypothermic storage of sperm in sturgeons: ideas, approaches, methods

'Classic' storage in open or unsealed containers. This approach, historically the earliest, and still prevalent (mainly among practicing fish farmers) is based on the idea that no special tricks for hypothermic storage are required because fish semen itself can be stored for at least several days in a refrigerator or on ice (ice bath) in open containers or vials plugged with a cotton-gauze swab. This method is described in many manuals and textbooks on fish farming with minor deviations in details [10], it is also often used during spawning campaigns. Indeed, in this state, the sperm retains its fertilizing ability for several hours and even days. There are sometimes rare reports of sperm preservation for 2-3 weeks. According to Aramli et al. (2017), who studied the hypothermic storage of undiluted sterlet sperm under 'classic' aerobic conditions, 30–40% of spermatozoa could be activated after 3 days

of storage, but no motile spermatozoa were observed after 6 days [14]. The results of such storage seem to be unpredictable and can only be compensated by a large number of batches. Perhaps the positive thing about this approach is that hypothermia could somehow play the role of selective factor for the best quality sperm batches.

Billard – DiLauro method. Büyükhatipoglu and Holtz (1978) revealed that filling containers with atmospheric air or pure oxygen helps to maintain fertilizing ability of undiluted rainbow trout milt for 9-15 days of storage. The best results were achieved while maintaining undiluted trout milt under pure oxygen (15 days of storage; 80% larvae hatching rate) and under ambient air (9 days; 94.7%) at +4 °C. Anaerobic conditions, dilution of milt or higher storage temperature impaired results [18]. In 1981, Billard proposed plastic bags filled with pure oxygen for hypothermic storage and transportation of rainbow trout semen [15]. Sperm survival and fertility were significantly higher after storage under pure oxygen compared to ambient air (18–21% O_2). The simplicity of this method and the ability to store the obtained fresh sperm from several hours to several weeks made it subsequently quite popular and widespread. It turned out that in this way it is possible to store the sperm of not only salmonids, but also cyprinids [32] and even sturgeons [35], whose sperm is different from that of teleost fish both in morphology and physiology. DiLauro et al. described preservation of Atlantic sturgeon *Acipenser oxyrinchus* sperm in plastic bags filled with oxygen on ice. Oxygen was changed daily. After storage for 5 and 17 days, 99% and 40% of spermatozoa, respectively, gained motility when activated [19, 20].

Actually, this method is recommended by FAO as the principal one for hypothermic storage of sperm in sturgeons. FAO Fisheries and Aquaculture Technical Paper №556 "Sturgeon Hatchery Manual" provides instructions for obtaining sturgeon sperm and its preservation in the hypothermic state, specifying the time during which sperm fertility is maintained depending on the duration of storage and initial quality of sperm batches. The sperm should be placed "*...into dry polyethylene packages or other dry containers filled with a 1:1 mix (oxygen : air) or (somewhat worse) with pure oxygen, where it is stored at the temperature of 0–0.5 °C (not higher that 3 °C) in a thin layer (not more than 0.5 cm thick)*" [8]. But one of the obvious disadvantages of this method is the condensation of evaporating water on the inner walls of the bag, and this fresh water contacts the sperm and forms foci of untimely activation and subsequent cell death. In addition, bags filled with gas as well as boxes for them filled with crushed ice or ice packs take up a lot of space which makes transportation inconvenient.

Conte method: storage in large volume syringes. Conte et al. (1988) came up with the idea of preserving sperm at +4 °C in 10–60 ml syringes partially filled with pure oxygen, which should be replaced every 12 hours. [22]. This storage method is compact (which is important for transportation), technically simple and more convenient compared to plastic bags, but still complicated by the need for regular oxygen replacement and depends on its source.

Preservation in Park – Chapman extender. Native sperm should be 4 times (1 : 3) diluted with the extender, which is the phosphate-buffered saline (pH 7.3-7.5, osmolality ~100 mOsm/kg) supplied with glucose or sucrose and Ca^{2+}/Mg^{2+} and K^+ cations in physiological concentrations [24]. The harvesting and storage of diluted semen is carried out using the same technique as according to the Billard – DiLauro method.

Storage in salt-free isotonic preservative solutions. This idea arose from attempts of applying the successful results of using salt-free preservatives for hypothermic storage of human and mouse sperm to sturgeons. In 1996, a group of researchers from the University of Yokohama proposed a new way to preserve human sperm at +4 °C in a salt-free aqueous solution containing nothing but glucose and bovine serum albumin [32]. This preserving medium named by the authors EFM (***e****lectrolyte* ***f****ree* ***m****edium*) made it possible to preserve human sperm in a viable state for at least 2 weeks [23, 29, 30, 32] which as a significant advance. Our preliminary studies also demonstrated that 52.0 ± 3.6% (n = 35) of spermatozoa restored motility after 2 weeks of hypothermic storage. At the same time, there was no significant difference (p = 0.11; n = 6) between the indices of DNA fragmentation before and after storage: 8.5 ± 2.5% and 11.2 ± 3.1% respectively, which supports the safety of this method [1]. From 2010 to 2013, a modified version of the technique was applied by us in 96 fertility treatment cycles, after obtaining voluntary written informed consent from patients. The fertilization rate was 78.0% and 74.9% of the embryos developed to the blastocyst stage. 34 healthy children were born as the result of 26 pregnancies [2].

A serious disadvantage of EFM is osmotic stress, from which cells suffer during dilution with a preservative and revitalization, and which leads to destruction of sperm membranes. To avoid this, we added trehalose to the preservative formula as 'osmotic buffer'. We named this modified preservative ISGT (***i****sotonic* ***s****olution of* ***g****lucose and* ***t****rehalose*) [3]. Comparing storage of epididymal spermatozoa of laboratory mice (*Mus musculus*) in ISGT vs EFM, we found that the addition of trehalose decreased the percentage of sperm with damaged membranes after 14 days of storage (1.4 ± 0.4% and 8.2 ± 1.2% respectively, p = 0.00, n = 16). At the same time, no differences were found in the ability of sperm to restore motility after storage (p = 0.57, n = 16) as well as in DNA fragmentation (p = 0.69, n = 12) for both preservatives. [3].

However, our first attempts to use EFM or ISGT media with the osmolality of ~320 mOsm/kg for hypothermic storage of sperm in sturgeons instead of the desired result led to failure – a rapid and irreversible loss of the ability to activate. This is due to taxonomic features in the anatomic structure and physiology of the male urogenital system in sturgeons. Consequently, osmolality of their seminal plasma is much lower than of blood plasma. Taking these features into account, we found empirically that the best preservation of sperm in sturgeons of different species could be achieved in salt-free solutions similar in osmolality to seminal plasma [4]. In 2012–2016, we developed the ISGT-80 preservative solution with osmolality similar to that of sterlet seminal plasma. We chose the sterlet (*Acipenser ruthenus*) because the osmolality of its seminal plasma is extremely low even for sturgeons – ~50–70 mOsm/kg [5, 26, 28]. After storage of sterlet sperm for 6 days at +2...+4 °C in ISGT-80, at least 50% of spermatozoa became motile while activated. After 6 days of storage, the percentage of spermatozoa with damaged DNA did not exceed 1.5%, and the proportion of spermatozoa with damaged membranes wes not more than 10% [5]. Sterlet eggs were inseminated with sperm stored for 10 days in ISGT-80 followed by the fertilization rate as high as 90% [6]. We also used a similar approach to develop experimental preservative solutions for hypothermic storage of sperm from Siberian sturgeon (ISGT-105) and stellate sturgeon (ISGT-150).

Conclusion

To date, despite some experimental advances and breakthroughs, hypothermic storage technologies are still not widely used in national sturgeon farms and hatcheries. The most pressing questions regarding hypothermic storage of sperm will remain open for a long time: (1) storage duration; (2) standardization of protocols i.e. a virtually predictable level of reproducibility; and (3) genetic stability while hypothermic storage technologies used, on which fertilization with subsequent development and eventually fish production depends. Nevertheless, the sporadic but ongoing studies both in Russia and abroad indicate the need for efficient technologies for the hypothermic storage of sperm in sturgeon breeding.

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