GENETICS

Viability of Postmortal Epididymal Mouse Spermatozoa during Long-Term Hypothermic Storage and Cryopreservation

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The study examined the effect of long-term hypothermic (4°C) storage of mouse carcasses on motility, cell membrane damage, *in vitro* survival and capacitation of epididymal spermatozoa before and after cryopreservation. It was shown that the number of spermatozoa with rectilinear forward motion decreased with increasing storage time. There were no significant changes in the total sperm motility and integrity of their plasmalemma. Pronounced effects of hypothermia and long-term storage of the mouse carcasses on cryocapacitation of spermatozoa during cryoconservation were demonstrated.

Key Words: *cryoconservation; hypothermia; postmortal epididymal spermatozoa; laboratory mouse; capacitation*

Long-term storage of reproductive cells is an actual problem of cryobiotechnology in medicine, agriculture, and ecology. Cryopreservation and storage of genetic material of rare domestic and wild animals in cryobanks are of special importance [1].

Intravital sperm is routinely obtained from ejaculate. In order to preserve the sperm of dead animals, the most mature spermatozoa are isolated from the epididymal tail. Although there are diverse methods of cryopreservation of mammal sperm described in vast literature, the routine technique is to isolate and preserve the sperm during 1-4 h postmortem [13]. It is difficult or even impossible to organize isolation and freezing the sperm during short postmortem period after sudden death of the animal. However, the data indicate that physiological activity and fertilizing ability of spermatozoa isolated from the epididymal structures after death of the mammal are preserved for a long period at low positive temperatures (4-6°C), so such spermatozoa can be used for cryopreservation [9,11,13]. Little data are available on maximal duration of postmortal spermatozoidal vitality and possibility of the use of such spermatozoa for cryopreservation. According to modern views, biological membranes are the most sensitive among the cell components to freezing and storage at low temperatures, so in addition to sperm motility, it is important to examine such parameters as their membrane permeability, capacitation, and the state of acrosome (acrosomal cap).

In this work, we examined *in vitro* the state of plasmalemma and acrosome, as well as motility and survival of cultured epididymal spermatozoa at various terms after animal death before and after cryopreservation.

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MATERIALS AND METHODS

The experiments were performed on 3-4-month-old NMRI mice randomized to groups (5 animals per group). The experiments were performed in strict adherence to the requirements for the research work on the experimental animals [4]. The mice were sacrificed by cervical dislocation. Mouse carcasses were placed into refrigerator at 4°C. Temperature measurements in the region of minor pelvis showed that the temperature of epididymides dropped *in vivo* to 4°C over 3.5 h.

The state of the epididymal sperm was examined on post mortem hours 24, 48, 72, and 120. Spermatozoa isolated from the non-cooled mouse carcasses immediately after the death (control 1) and spermatozoa isolated after cooling the carcasses for 3.5 h (control 2) were used for comparison.

The sperm was isolated as follows: the carcasses were opened, the epididymal tail was isolated and placed in 0.05 ml Dulbecco's phosphate buffered saline supplemented with 10% mouse blood serum to determine vitality of non-frozen spermatozoa. Alternatively, it was placed in cryoprotectant medium for cryopreservation. Then, the epididymal tail was cut into 3-4 segments, delicately pressed with plane forceps to mechanically squeeze the spermatozoa from the epididymal lumen. The resulting suspension was collected with a pipette. The remaining fragments of the organs were washed two times in 0.05 ml DPBS. In some cases with high concentration of spermatozoa, the suspension was additionally diluted with the same medium.

The following solution was used for cryopreservation of the sperm: sucrose (0.29 M), DMSO (0.17 M), HEPES (9 mM at pH 6.8). The medium was supplemented with 5% egg yolk before use. Aliquots of sperm suspension (0.05 ml) were placed in straws of 0.2 ml and frozen in liquid nitrogen vapor at -80 to -90°C, thereupon they were placed in liquid nitrogen for storage. Thawing was performed in water bath at 40°C to the ice melting stage. Then the suspension was diluted with double quantity of DPBS supplemented with mouse blood serum for 1.5 min by three stages.

Vitality of spermatozoa was assessed by several parameters: motility, survival, and plasmalemma integrity.

To analyze sperm motility and survival, the sperm suspension (native or frozen-thawed) was placed in an incubator at 37°C, 100% humidity, and 5% CO_2. After 10-min culturing, the relative cotent of motile spermatozoa in the sample was assessed. The number of spermatozoa with normal rectilinear forward motion (RFM) was also counted. In addition, the total number of motile spermatozoa demonstrating any king of motion (oscillatory and rotary, also refereed as "in manege" motion) was termed as total motility (TM) and counted. Then, sperm motility was assessed every hour of incubation to determine survival, *i.e.* the time when motile spermatozoa could be detected after they have been introduced into the culturing medium *in vitro*.

The integrity of spermatozoon plasmalemma was evaluated after 10-min culturing at 37°C by permeability of the cell membrane for fluorescent dye ethidium bromide [2]. A LYUMAM-IZ (LOMO) luminescent microscope was used to count fluorescing spermatozoa (λ=600 nm), which reflected the relative content of cells with damaged membrane.

Analysis of capacitation and acrosome state was based on the use of fluorescent antibiotic chlortetracycline CTC [16]. This method determines sperm capacitation, *i.e.* the final step of spermatozoon maturation outside the male reproductive tract based on changes in plasmalemma composition (decrease in the cholesterol-lipid ratio, changes in phospholipid composition, increase in cell membrane fluidity, phosphorylation of tyrosine residues of membrane proteins, *etc*.). Capacitation yields the spermatozoa capable for the acrosome reaction (release of the acrosomal enzymes after shedding the acrosomal cap triggered by contact with oocyte plasmalemma) resulting in fertilization.

To analyze the state of acrosomal machinery, 5 μl sperm suspension was mixed with 5 μl 0.025% CTC solution. Staining was performed for 20 sec, thereafter the cells were stabilized by addition of 0.2 μl 2.5% glutaraldehyde. Distribution of fluorescent intensity over spermatozoon was assessed in green light (λ=470-520, ×900). Homogeneous fluorescence of the spermatozoon head was interpreted as the absence of capacitation. In contrast, the cells with shaded postacrosomal region were assessed as capacitated. Spermatozoa with non-fluorescent head were regarded as cells with shed (lost) acrosomal cap.

The data were processed statistically using Excel and Sigma-Plot software.

RESULTS

Effects of cooling and storage time of the mouse carcass at 4°C on physiological parameters of the sperm. Below are the data on mouse spermatozoa motility (TM and RFM) obtained after storage of the carcasses at 4°C up to 5 days (Table 1). The total number of motile spermatozoa isolated from cooled carcasses 3.5 h postmortem did not differ from that obtained immediately after death. When cooled carcasses were stored up to 5 days, the total number of motile spermatozoa decreased by 20% ($p<0.01$). The storage time affected the character of spermatozoon motility. During the first postmortem hours, almost 90% motile cells demonstrated the normal type of motion (RFM), while on storage day 5 about 60% cells were involved in

the oscillatory motion. The ability of spermatozoa to RFM rapidly waned even to the end of the storage day 1. During this period, the share of spermatozoa with RFM dropped 2-fold from 48 to 23% ($p<0.05$). After storage for 48-120 h, RFM was below the control by 3 times approximately (Table 1). Correlation between RFM and storage time was -0.54±0.18.

Similar data were obtained in studies of low-temperature postmortal storage of epididymides of mice [12], sheep [11], deer [8,9], dogs [15], and feline species [6,10]. These studies revealed a negative effect of postmortal storage of the epididymides on RFM of native and frozen-thawed spermatozoa. The degree and character of this effect was diverse for different species and various conditions of hypothermic storage (isolated gonads or the gonads within the carcasses). Our data and those of other studies performed on various species showed that RFM index decreased with increasing the duration of hypothermic storage at low positive temperatures. An exception is the report [14] on significant changes in RFM of bovine spermatozoa in contrast to pronounced drop of TM index during hypothermic storage of the gonads for 72 h.

In our study, hypothermic storage of mouse carcasses at 4°C for 5 days did not significantly elevated the number of epididymal spermatozoa with damaged membranes, which was seen from the absence of fluorescent spermatozoa after staining with ethidium bromide (Table 2) revealing no changes in membrane permeability for the dye. It is common knowledge that elevation of plasmalemma permeability is a step preceding irreversible alterations in the cells [5]. A low number of spermatozoa with damaged membranes observed in this study suggests that unfavorable physiological changes in spermatozoa going on during the examined terms of hypothermic storage can be reversible, so in the following (under the certain conditions of the storage) the postmortal sperm of higher quality can be obtained.

An important index of sperm functional state is survival of spermatozoa in culture *in vitro* at 37°C, which depends on consorted work of various cellular systems, whose most important member is cell energy machinery. The mean survival of spermatozoa isolated from the carcasses after 3-5 day storage at 4°C was greater than that of the cells isolated immediately after cooling the carcasses (Table 2). At first look, this fact seems paradoxical. However, the reverse relation between the mean velocity of spermatozoon motion and their life span is well documented [3]. It is possible that decrease of motility (reflected by RFM index) during long-term storage of the epididymides could negatively affect the spermatozoon life span [3].

Effects of cooling and time of mouse carcass storage at 4°C on physiological indices of cryopreserved sperm. Table 3 shows physiological indices of epididymal spermatozoa cryopreserved at various terms postmortal. Comparison of the control 1 and control 2 indices showed that preliminary cooling the mouse carcasses before isolation of spermatozoa for the following cryopreservation did not degrade the quality of the frozen-thawed sperm. Analysis of motility of the cryopreserved sperm derived from non-cooled and cooled carcasses revealed no significant differences in the TM and RFM indices in both groups. Similarly, there were no significant differences between spermatozoon survival and the plasmalemma state in these groups.

Prolongation of the storage time of cooled mouse carcasses before isolation and freezing the sperm produced no effect on TM of the thawed spermatozoa, but pronouncedly decreased their RFM index. One-day storage before cryopreservation decreased RFM of the frozen-thawed sperm 2-fold ($p<0.05$). The corresponding RFM values for 48- and 72-h storage were 3- ($p<0.05$) and 6-fold lower ($p<0.01$) respectively.

Survival of cryopreserved spermatozoa isolated from cooled carcasses after different storage time (from 3.5 to 72 h) did not significantly differ. How-

TABLE 1. Effect of Long-Term Storage of Mouse Carcasses at 4°C on Rectilinear Forward Motion and Total Motility of Spermatozoa (*M±m*)

Storage time, h	RFM, %	TM, %
0	52±12	59±12
3.5	48±10	55±12
24	23±13*	50±7
48	16±10*	42±13
72	18±6*	42±4
120	17±4*	44±6.5

Note. Here and in Table 2: *$p<0.05$ compared to control 2 (3.5 h).

TABLE 2. Effect of Long-Term Storage of Mouse Carcasses at 4°C on Spermatozoon Survival *In Vitro* at 37°C and on Membrane Permeability (Spermatozoa with Intact Plasmalemma)

Storage time, h	Survival, h	Undamaged spermatozoa, %
0	4.7±0.60	74.5±6.4
3.5	4.3±0.50	79.0±7.0
24	4.0±0.60	65.6±7.3
48	4.3±1.00	65.0±10.0
72	6.0±1.05*	65.0±11.0
120	5.5±0.70*	60.0±11.3

TABLE 3. Effect of 4°C Storage Time before Isolation and Cryopreservation (-196°C) on Physiological Indices of Frozen-Thawed Epididymal Sperm (*M*±*m*)

Index	Sperm isolated from non-cooled carcasses (C1)	Sperm isolated from the cooled carcasses with different storage time			
		3.5 h (C2)	24 h	48 h	72 h
RFM, %	19±9	23±3	12±8*	7±3*	2±2+*
PM, %	28±4	29±4	32±11	29±12	29±4
Spermatozoon survival *in vitro*, h	3.7±0.4	4.5±0.6	4.3±1.3	4.6±0.6+	4.9±1.0+
Spermatozoa with damaged plasmalemma (test with ethidium bromide), %	34±12	41±11	45±4	41±7	47±4
Capacitated spermatozoa (CTC test), %	11±4	3±1+	5±2+	12±4*	15±5*
Spermatozoa with shed acrosome (CTC test), %	0	0	0	1.5±1.0+*	3±3+*

Note. C1, control 1; C2, control 2; $p<0.05$ in comparison with *C2 or +C1 (Student *t* test).

ever, the spermatozoa frozen after 48- or 72-h hypothermic storage demonstrated significantly greater life span *in vitro* in comparison with sperm isolated from non-cooled carcasses ($p<0.05$). Probably, this fact can be explained by adaptation of spermatozoa to low temperatures before freezing.

Capacitation of the spermatozoa is necessary for fertilization of the eggs. Normally, capacitation is triggered by female reproductive tract secretions. Cryopreservation of the sperm can provoke a capacitation-like reaction called "cryocapacitation" [7], which is an undesirable phenomenon, because these spermatozoa are predisposed to premature acrosome reaction and to the loss of fertilizing potency. The studies in this direction were carried out on intravital ejaculated sperm, which is most widely used in practice. Possible cryocapacitation of the postmortal sperm is still an unexamined phenomenon.

In our experiments with epididymal sperm isolated and frozen immediately after mouse death (control 1), we found 8-14% (the mean value 11%) share of the capacitated spermatozoa. Preliminary cooling of carcasses before cryopreservation significantly moderated the effect of cryopreservation on cryocapacitation. For example, the sperm isolated from the cooled carcasses demonstrated 3.5-fold smaller capacitated spermatozoa (3%) after cryopreservation in comparison with control 1. After prolongation of the storage time to 48 h and more, the number of capacitated spermatozoa again increased to 12-15% ($p<0.05$).

The sperm isolated and cryopreserved within the postmortal period of less than 24 h had no spermatozoa with shed acrosome. Insignificant number of the spermatozoa with shed acrosomal cap was established only in the specimens cooled for 48 h and more post mortem.

Based on the complex of physiological parameters (motility, survival, capacitation, and integrity of acrosome and plasmalemma) this study concludes that the epididymal spermatozoa of the dead mice maintain vitality at low positive temperature of 4°C for a rather long period of no less than 5 days. The hypothermic storage dramatically affects the process of sperm capacitation not previously examined in relation to the postmortal sperm. The capacitation index should be taken into consideration in the following work aimed to improve the methods of cryopreservation of the postmortal sperm.

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