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ULTRA-LOW CONCENTRATIONS OF BENZIMIDAZOLE DERIVATIVES CAN INCREASE BULL AND HORSE SEMEN RESISTANCE AT CRYOPRESERVATION AND UNDER THE INFLUENCE OF DAMAGING FACTORS

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Abstract

One of the possible ways to improve sperm cryopreservation is to find how to increase the resistance to damaging effects of low temperatures. Here we summarize our findings on the bull and stallion semen cryoresistance as influenced by ultra-low concentrations of biologically active substances, the ethyl-1-benzimidazol-2-yl-sulfanyl, 2-ethylsulfanyl-benzimidazol-1-yl and 2-benzimidazol-1-yl-acetic acid. It was found that these substances increased survivability of bull semen during storage in lactose-citrate semen extender. The best motility and vitality of sperm after freezing and thawing was observed when sperm was diluted by extender with added 2-benzimidazol-1-yl-acetic acid in the ultra-low concentrations of 10^{-13} to 10^{-15} M. The viability of sperm to 10 % motility was 73 % higher as compared to control. Similarly, freezing equine sperm in extender supplemented with 2-benzoimidazol-1-yl-acetic acid at 10^{-13} M was more effective: the semen survival after freezing and thawing was 8.1 % ($P < 0.01$) higher than that in the control, and the intactness of acrosome was 1.9±0.63 % higher ($P < 0.05$). 2-Benzimidazol-1-yl-acetic acids also improved semen vitality at 40 °C when different osmolarity and after cold shock. It can be assumed that the observed phenomenon is likely due to the protective effect of 2-benzimidazole-1-yl-1-acetic acid to plasma membrane and the mitochondria membrane structure of spermatozoa. Study of respiration in bovine sperm after freezing and thawing confirmed this assumption. Indeed, dinitrophenol almost equally increased cell respiration despite the presence or absence of 2-benzimidazole-1-yl-1-acetic acid in the semen extender while succinate, which penetrates through the damaged membranes, had less stimulating effect when 2-benzimidazole-1-yl-1-acetic acid added. The studies suggested the hypothesis that benzimidazole, a biologically active substance, at ultra-low concentrations can bind to a receptor on the sperm outer membrane resulting in the cell membrane restructuring. At the same time, the changes in viscosity of water associated with the membrane proteins may occur due to hydrogen bonds between water molecules and acid residues of benzimidazole molecules. As a result stability of the membrane structures to damaging effect of varying osmotic pressure increases. Possibly crystal formation of water associated with the cell membranes is decreasing during freezing that also reduces the damaging effect.

Keywords: sperm, freezing, benzimidazole, ultralow concentrations, cell membrane, mitochondria, bulls, stallions

Despite widespread use of cryopreserved sperm in breeding various types of farm animals, the death of up to 40-50 % of germ cells after freezing remains a problem for the practice of artificial insemination, so the search for ways to make spermatozoa resistant to the damaging effect of low temperatures is still relevant [1-5].

Nonspecific increase in cell resistance under the influence of chemicals of different nature in subthreshold doses and concentrations, several orders of magnitude lower than the sub-toxic, has been described for a long time. In the

domestic literature, early publications on this topic include studies that confirmed an increase in the time of spermatozoa survivability under the influence of subthreshold doses of a number of agentsю e.g. drugs, urea, inhibitors of metabolism, etc. [6-11]. Later, substances with the ability to cause an increase in cell resistance at concentrations several orders of magnitude lower than the subtoxic levels were found. Thus, it has been shown that benzimidazole and its derivative dibasol in concentrations of 10^{-3}-10^{-11} M promotes resistance of cells and tissues to the damaging effects of low and high temperatures [12-14].

In the last 20 years the attention of researchers has been attracted by the phenomenon of the effectiveness of ultra-low doses (ULD, 10^{-12}-10^{-15} M) substances with respect to biological objects. First of all, the reason is that many compounds in ULD can cause response reactions that are comparable and even more significant than at substantially higher concentrations [15, 16]. Attempts to explain the mechanism of the biological effect of physical and chemical factors in the ULD [17-21] have not led to a single opinion to date. Nevertheless, ULD in a number of cases found a successful application in medicine [22-24] and veterinary medicine [25, 26].

We first studied the effect of benzimidazole derivatives on the resistance of bull and stallion spermatozoa to the damaging effect of low and ultra-low temperatures during cryopreservation, and it was shown that the greatest activity and safety were noted when 2-benzimidazole-1-yl-1-acetic acid of at an ultra-low concentration of 10^{-12}-10^{-15} M was introduced into the medium for semen freezing.

The aim of this work is to study the effect of low and ultra-low concentrations of benzimidazole derivatives on the survivability of spermatozoa during dilution, freezing and thawing, their resistance to cold shock in the media with different osmolarity.

Technoque. Benzimidazole derivatives were synthesized at the Department of Organic Chemistry of the St. Petersburg State Agricultural University. Used concentrations of the substances in the diluent were 10^{-3}, 10^{-5}, 10^{-11}, 10^{-13}, and 10^{-15} M.

The experiments used sperm of black-and-white bulls (n = 11) of Leningrad type (FGUP Nevskoe, Leningrad region) and Trakehner, Hanoverian, Arabian and Holstein stallions (n = 10) (OOO Cowboy, Malanichevs farm and private owners, Leningrad region). The bull sperm had an initial mobility of 5-6 points (because of this values the samples were not allowed to freeze for production purposes; when measuring respiration rate, samples with greater mobility were used), stallion sperm had 7-8 points. To compare the experiment options, each ejaculate was divided into equal parts.

When determining the resistance of bull spermatozoa to changes in osmotic pressure and cold shock, an aqueous lactose solution was a diluent. Control was a solution with an osmolarity of 336 mosm/l, containing 11.5 g of lactose per 100 ml of water; to increase or decrease osmolarity in the series 246, 276, 306, 336, and 366 mosm/l, the amount of lactose was increased or decreased (by 1.02 g per 30 mosm/l). The procedure for sample freezing corresponded to the interstate standard (GOST 26030-2015) [27]. Cold shock of bull spermatozoa was caused by a decrease in the temperature of diluted semen from 20 to 4 °C for 2 min.

The sperm of the stallions were diluted in Kenney medium (49 g of D-glucose, 24 g of dried milk, 40 mg of gentamicin, 1 liter of distilled water) in a volume ratio of 1:3, then centrifuged for 8 min at 600 g. The spermatozoa residue was suspended in Kenney medium and diluted 100 million cells/ml concentration, the semen was packed into 0.5 ml straws, cooled at 4 °C for 90 min and

frozen in liquid nitrogen vapor for 12 min at 110 °C, then lowered in liquid nitrogen. The stallion sperm was thawed at 37 °C for 1-2 min.

The volume, number and mobility of spermatozoa (complete absence as 0 points, 100 % as 10 points) were assessed by conventional methods. Morphology and condition of the acrosome cap were studied with phase-contrast light microscopy. The time of the survivability of the bull sperm was expressed in hours to the preservation of 10 % of mobile cells and to the complete loss of mobility.

The respiratory activity of the cells was determined according to the description [28] on a polarograph LP 7 (Czech Republic) with a Clark platinum electrode. The incubation medium was 6 % glucose, in which sperm (50-100 million spermatozoids per ml) was successively added to final concentration, potassium succinate was a substrate (1.0×10^{-3}-2.5×10^{-3} M), and proton ionophore 2.4-dinitrophenol (DNP, 2.5×10^{-5} M) served as classical uncoupler of cellular respiration and phosphorylation.

Damage to the plasma membranes of stallion spermatozoa was assessed using Sperm VitalStain dye (Nidacon International AB, Sweden). Staining was performed in Eppendorf tubes (50 μl of sperm were mixed with 50 μl of dye) and smears were made on slide glasses. The preparations were examined with ×100 magnification (lens) and oil immersion, counting at least 200 cells in each sample (white cells mean no damage, red or pink were spermatozoa with damaged membranes).

The Axio Imager visualization system (Carl Zeiss Microscopy GmbH, Germany) was used for microscopy.

Data was processed in SigmaPlot 12.5 (Systat Software Inc., USA) and Micrisoft Excel programs. We performed a general statistical analysis and an estimation of the average difference between samples with pairwise coupled variants. The differences were considered statistically significant at $P < 0.05$. The tables show the mean (X) and standard error of the mean ($\overline{x}$).

Results. One of the possible approaches to the practical solution of the preservation problem of cryopreserved spermatozoa is to find ways to increase their resistance to the damaging effect of low temperatures. To date, enough data have been accumulated on the effect of cooling on cells [29, 30], which allow us to draw definite conclusions about the mechanisms of damage and approaches to preventing these damages. It is possible to distinguish two types of damage to cellular structures that occur during the action of cold, i.e. associated with cooling prior to freezing and resulting from crystal formation during freezing. Among the factors influencing the survival of cells and tissues during cryopreservation, the cold shock [31] and fluctuations in osmotic pressure during dilution, freezing and thawing of sperm are distinquished.

When comparing the time of survivability of bull spermatozoa at 20 °C in the presence in the diluent medium of three benzimidazole derivatives in concentrations of 10^{-3}-10^{-15} M, it was found that the survival of bull spermatozoa during storage in the lactose-citrate medium was increased. The greatest positive effect was given by ethyl-1-benzimidazole-2-yl-sulfanyl at a concentration of 10^{-5} M (46 % excess), 2-ethyl-sulfanyl-benzimidazole-1-yl at a concentration of 10^{-11} M (59 %) and 2-benz-imidazol-1-yl-1-acetic acid in concentrations of 10^{-11} M, 10^{-13} M and 10^{-15} M (by 88-90 %).

Comparison of the effect of the most effective concentrations of the studied derivatives at an elevated plus temperature (40 °C) (Table 1) showed that the greatest activity and survivability of bull spermatozoa after freezing-thawing was due to the presence in the medium for the semen dilution of 2-benzimidazol-1-yl-1-acetic acid in ultra-low concentrations of 10^{-13}-10^{-15} M, or

only 4-6 molecules of the test substance per spermatozoon. In this variant, the percentage of surviving spermatozoa (activity) was significantly higher (by 8-13 %, $P < 0.01$) than in the control. The time of survivability until the mobility of 10 % of the spermatozoa increased by 73 %, and the time until the mobility ceased completely exceeded the control by 85-90 %.

1. Survivability of bovine spermatozoa at 40 °C after freezing and thawing in a medium with ultra-low concentrations of benzimidazole derivatives ($n = 7$, $X \pm \bar{x}$)

Option	Activity after thawing, point	Survivability time, hour	
		up to 1 point (10%)	up to 0 point
Control	2,6±0,28	1,5±0,18	2,0±0,19
Benzimidazole derivative, M:			
ethyl-1-benzimidazol-2-yl-sulfanyl, 10^{-5}	3,2±0,01	2,3±0,22*	2,9±0,21**
2-ethylsulfanyl-benzimidazol-1-yl, 10^{-11}	3,0±0,23	2,0±0,23	3,2±0,18**
2-benzimidazol-1-yl-1-acetic acid, 10^{-11}	3,2±0,38**	2,0±0,21	2,9±0,24**
2-benzimidazol-1-yl-1-acetic acid, 10^{-13}	3,9±0,26***	2,6±0,21***	3,7±0,21***
2-benzimidazol-1-yl-1-acetic acid, 10^{-15}	3,4±0,07***	2,6±0,09***	3,8±0,15***

N o t e. Bull sperm with initial activity of 5-6 points were used.
*, **, *** Differences with the control are statistically significant, respectively, at $P < 0.01$.

Similar results were obtained by freezing and thawing of stallion sperm in a medium with 10^{-13} M 2-benzimidazol-1-yl-1-acetic acid. The number of mobile intact cells was 15.2±3.49 million/ml greater ($P < 0.01$) than without supplement, the survivability of spermatozoa after freezing-thawing increased by 8.1 % ($P < 0.01$), the preservation of the acrosome was 1.9±0.63 % higher ($P < 0.05$).

2. Survivability of bovine spermatozoa at 40 °C and different osmolarity under the influence of 2-benzimidazole-1-yl-1-acetic acid (10^{-13} M) ($n = 11$, $X \pm \bar{x}$)

Osmolarity, mosm/l	Mobile cells, %					
	after dilution		after an hour at 40 °C		after two hours at 40 °C	
	control	experiment	control	experiment	control	experiment
366	$28\pm4,8^{a}$	$42\pm5,8^{a}$	$10\pm0,9^{g}$	$14\pm1,6^{g}$	0	0
336	$60\pm3,2^{A}$	$60\pm3,2^{B}$	$29\pm3,7^{d}$	$41\pm3,3^{d}$	$11^{i}\pm2,4$	$26^{i}\pm3,0$
306	$24\pm2,4^{b}$	$40\pm4,4^{b}$	$11\pm1,6^{e}$	$19\pm2,7^{e}$	0	6±1,6
276	$15\pm2,2^{c}$	$31\pm4,0^{c}$	0	12±1,5	0	0
246	4±2,4*	20±3,2*	0	6±1,8	0	0

N o t e. Bull sperm with initial activity of 5-6 points were used.
* Differences in variants aa, bb, cc, dd, ee, ii, Aa, Ab, Bb and Ba are statistically significant at $P < 0.01$, in gg - at $P < 0.05$.

2-Benzimidazole-1-yl-1-acetic acid at the same concentration of 10^{-13} M positively influenced the stability of bovine spermatozoa as the osmolarity changed (Table 2). Immediately after dilution at room temperature (20 °C) in osmolarity isotonic for bovine sperm (336 mosm/l), 2-benzimidazole-1-yl-1-acetic acid did not affect the cell activity compared to the control. However, after 1 and 2 hours after storage (at 40 °C) in the presence of an additive, the motility of the spermatozoa in the isotonic medium turned out to be 12 % and 15 % higher, respectively ($P < 0.01$), than in the control. With a decrease or increase in the osmolarity by 30 mosm/l to the isotonic level, the proportion of mobile cells in the sperm decreased immediately upon dilution (see Table 2), but in a medium with 2-benzimidazol-1-yl-1-acetic acid (10^{-13} M) only by 18-20 % compared to 32-36 % in the control. In hypoosmotic conditions, a complete sperm death was observed after 1 h in the control, while in the experiment 6-12 % of the cells retained motility. After 1 h in hyperosmotic conditions (366 mosm/l), the percentage of mobile spermatozoa in the experiment was significantly higher (by 14 %, $P < 0.05$) compared to the control.

Phase-contrast microscopy revealed no morphological differences in the acrosome and sperm flagellum between the experimental and control variants

immediately after dilution, whereas different osmolarity of the medium led to a change in the safety and viability of the spermatozoa (see Table 2). After a cold shock in the control, 40 % of the spermatozoa lost their mobility, while in 10^{-13} M 2-benzimidazole-1-yl-1-acetic acid, the motility of spermatozoa decreased by 26 %. It can be assumed that the observed increase in cell resistance to osmotic influence and cold shock is associated with the protective effect that the benzimidazole derivatives have on the membrane structures of spermatozoa.

As is known, the mobility and time of sperm survivability depend on energy supply, respiration and phosphorylation, and are directly related to the functional state of the mitochondria. The mitochondrial membranes are most sensitive to damaging factors [28]. Succinate enhances cellular respiration, penetrating only through the damaged plasma membrane, and DNP serves as an uncoupler of tissue respiration and oxidative phosphorylation [28]. In our experiments (Table 3), the stimulation of the respiration of bovine spermatozoa by succinate after freezing and thawing in the presence of 2-benzimidazole-1-yl-1-acetic acid (10^{-13} M) was lower, and with DNP was higher than in control, that indicates a better energy supply when using an additive. This confirms the assumption that the ultra-low concentrations (doses) of the benzimidazole derivatives contribute to an increase in the stability of the spermatozoa membrane at an ultra-low temperature.

3. Change in respiratory rate in bovine spermatozoa under the influence of 2-benzimidazole-1-yl-1-acetic acid (10^{-13} M) **during freezing and thawing** ($X \pm \bar{x}$)

Sperm	Activity, point	Respiration, nmol O_2/ min		
		rate	stimulation	
			succinate K	DNP
Freshly diluted	7.0-8.0	130.0±1.56	1.06±0.012	2.20±0.050
After freezing and thawing:				
control	4.0-5.0	77.0±3.82	2.03±0.187	1.57±0.029
experiment	4.5-6.0	88.0±5.03	1.47±0.730*	1.84±0.021*

N o t e. DNP - 2.4-dinitrophenol.
* Differences with the control are statistically significant at $P < 0.01$.

It is known that the cooling and freezing of spermatozoa results in the release of K^+ ions into the medium, which adversely affects cell vitality [31]. Benzimidazole derivatives serve as plasma membrane H^+/K^+-ATPhase inhibitors and prevent excess K^+ release. Apparently, interacting with the receptor on the outer cell membrane, benzimidazole derivatives cause a cascade rearrangement of the membrane structures. At the same time, the viscosity of water changes due to the occurrence of hydrogen bonds between molecules with the formation of clusters. As a result, the resistance of membrane to the damaging effect of osmotic pressure fluctuations occurring during freezing and thawing can increase. Besides, it is possible that when the water freezes, the character of crystal formation changes towards decreasing crystal size, which reduces their damaging effect. The absence of a pronounced effect of the substances at intermediate concentrations agrees with the classical theory of R.P. Stephenson [32-34], according to which the maximum effect is achieved by binding the ligand to only a small part of the receptors.

So, when ultra-low concentrations (10^{-13}-10^{-15} M) of 2-benzimidazole-1-yl-1-acetic acid was introduced into the medium used in freezing bull and stallion sperm, the spermatozoa were the most active and safe after freezing and thawing, cold shock, elevated temperature (40 °C) and when osmolarity changed (as it could be concluded from the time of survivability and the proportion of cells that remained mobile). Dinitrophenol almost equally strengthened cellular respiration in the experiment and in the control, while succinate, which penetrates through damaged membranes, had less stimulating effect in the presence of 2-

benzimidazole-1-yl-1-acetic acid. The observed effect of 2-benzimidazole-1-yl-1-acetic acid is presumably associated with its protective influence on the plasma membrane and the membrane structures of mitochondria in spermatozoa due to interaction with the receptor on the outer membrane, and, possibly, may be caused by the effect on the state of water molecules associated with membrane proteins.

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