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VITAMINS - AS POSSIBLE COMPONENTS OF CRYOPROTECTIVE MEDIUMS FOR PRESERVATION OF BOAR SPERM

Ion BALAN, Vladimir BUZAN, Nicolae ROŞCA, Gheorghe BORONCIUC, Iulia CAZACOV, Melania BUCARCIUC, Ion MEREUŢA, Nadejda ZAICENCO, Alexandru DUBALARI, Nicolai FIODOROV, Irina BLÎNDU

Institute of Physiology and Sanocreatology, 1 Academiei Street, MD 2028, Chişinău, Republic of Moldova

Corresponding author email: vladimirbuzan@yahoo.com

Abstract

This paper presents some methods and approaches for cryopreservation of boar semen. Despite the long history of experimental research, most of these methods are still not effective enough for routine use in pig farms. The aim of our research was to identify the most effective traditional methods of cryopreservation of reproductive material and to determine the effectiveness of the inclusion of vitamins in the composition of synthetic mediums for dilution and freezing of boar sperm of Landrace breed. Through physiological and biochemical methods it has been established that vitamins of different nature and structure can be included in the composition of cryoprotective mediums, depending on the purpose of the study and their physicochemical properties. Experimental studies have revealed that the tested vitamins have a cryoprotective effect consisting in increasing of the absolute survival rate of reproductive cells when keeping them at room temperature. One of the mechanisms of the cryoprotective effect may be inhibition of the process of lipid peroxidation, which is confirmed by a decrease in the amount of malondialdehyde (MDA). As a result of the study of included vitamins, it was concluded that vitamin E (tocopherol) prevail from the tested vitamins. The antioxidant effect of vitamins, presumably, may be due to their inclusion in the composition of the active center of peroxidation enzymes that decompose reactive oxygen species.

Key words*: vitamins, cryoprotective mediums, sperm, antioxidant effect.*

INTRODUCTION

Studies of recent years (Нарижный et al., 2001; Нарижный et al., 2014; Яковлев et al., 2007) show that among the fundamental problems of livestock biotechnology is an increase in the efficiency of animal reproduction by maximizing the use of breeding producers. In addition to biological indicators of sperm, there are factors that affect the effectiveness of insemination of frozen-thawed sperm. It is known that cryopreservation stimulates lipid peroxidation in boar semen. Thus are damaged the important structures of spermatozoa involved in the regulation of metabolism and fertilization processes of oocytes. This may be one of the reasons for the decline in the fertilizing ability of frozen-thawed boar semen.

According to (Милованов, 1962; Нарижный et al., 2014) among the substances included in the protection system, antioxidant membrane-protective role of vitamin is substantiated. Their properties are manifested by a number of complex effects at all levels of organization - from membrane formations to the organism as a whole. It was found that the use of antioxidant vitamins contributed to increasing the mobility of boar spermatozoa immediately after thawing and increased their survival at 39°C. In addition, their introduction has contributed to improving the preservation of spermatozoa membrane structures during freezing. The cryopreservation method is widely used both in modern reproduction biotechnology and in solving problems of biodiversity conservation.

One of the main technological stages of sperm cryopreservation is its dilution with synthetic mediums, the improvement of which allows to increase the efficiency of long-term preservation of biological objects at ultra-low temperatures. Given the fact that in any direction of research is constantly searching for better methods to improve product quality, in these studies, during freezing and thawing of

sperm, antioxidants vitamins were tested because the question of increasing the fertilizing ability of frozen-thawed sperm is relevant.

MATERIALS AND METHODS

As an experimental material was used the sperm of Landrace breeding boars, which were kept in the conditions of the State breeding enterprise "Moldsuinhibrid" in accordance with zoo-veterinary requirements. Sperm motility was evaluated by viewing in an "Ampleval" microscope of the manufacturer Carl Zeiss, at 200-fold magnification. The absolute survival rate of spermatozoa was calculated using the generally accepted method (Милованов, 1962). Glucose-chelate-citrate medium (GCC) was used as a base medium (BM) for dilution and storage of sperm. In the experimental variants has been studied the efficacy of different vitamin concentrations obtained by the method of counter series by Milovanov V.K. (Милованов, 1962). Determination of the content of one of the products of lipid peroxidation, malonic dialdehyde, was carried out according to the method of Vladimirov Y.A. and A.I. Archakov (Владимиров, 1972), in the modification of the researchers of our laboratory, which consists in determining the concentration of gametes of the studied samples, instead of protein, and clarifying the calculation formula. At the same time, the concentration of malonic dialdehyde was determined in nanomoles per 10^9 cells, taking the molar attenuation coefficient equal to $1.56*10^{-5}$ $M^{-1}\cdot cm^{-1}$.

Statistical processing of the digital material was carried out by analyzing the data of the computer program Microsoft Excel 2010, using the Student's t-test.

RESULTS AND DISCUSSIONS

At technological processing of sperm, diluting it with synthetic mediums and storing it in a cooled or deep-frozen state, occurs significant structural and biological damage of spermatozoa, which leads to disruption of plasma membrane permeability and the exit of a number of enzymes and other components of cellular metabolism from spermatozoa, which significantly reduces sperm fertility (Нарижный et al., 2001; Нарижный et al., 2014). As our working hypothesis, we used the properties of vitamins, which are biologically active substances (Combs et al., 2012; Овчинников, 1987), in connection with which they can influence the process in the composition of mediums for diluting and freezing the sperm of Landrace breeding boars.

It is well known that the reaction of free radical oxidation are of particular importance in the regulation of the functional activity of spermatozoa. This is due to the fact that the marked reactions are a necessary stage of various metabolic processes, the cause or consequence of pathological changes at the cellular level. Excessive accumulation of lipid peroxidation products, triggered by the presence of reactive oxygen species, leads to a change in the intermolecular interactions of the lipiprotein complex of biological membranes, an increase in their permeability to ions and water, a decrease in the activity of membrane-bound enzymes, and the appearance of transmembrane defects that can cause spermatozoa destruction. Reinforcement of lipid peroxidation can occur under the influence of various environmental factors, including temperature changes.

In our studies, to reduce the intensity of lipid peroxidation during the process of diluting and storing of boar semen under hypothermal conditions, was determined the optimum concentration and efficiency of using vitamins as antioxidants in synthetic mediums. The results of experimental studies using vitamin E are presented in Table 1.

Table 1. The effect of vitamin E on the physiological parameters of diluted sperm of breeding boars

№	Concentration of vitamin, mg/ml	Motility after dilution, points	Absolute survival rate, conventional units	
			after 12 hours	after 24 hours
1	BM - control	7.1 ± 0.23	85.2 ± 1.34	163.2 ± 6.84
2	BM + 0.25	7.4 ± 0.13	88.8 ± 2.50	170.4 ± 2.68
3	BM + 0.5	7.6 ± 0.11	91.2 ± 1.90*	177.6 ± 2.68
4	BM + 1.0	7.6 ± 0.11	91.2 ± 1.90*	175.2 ± 3.29
5	BM + 2.0	7.0 ± 0.08	84.0 ± 0.11	163.2 ± 3.29
6	BM + 4.0	7.0 ± 0.08	84.0 ± 0.11	156.0 ±4.24

* The differences are statistically authentic

From the data of Table 1 it follows, that from the tested concentrations in the range of 0.25-4.0 mg/ml the third variant of the experiment turned out to be optimal, when the content of the tested vitamin was 0.5 mg/ml. Sperm motility after dilution was 7.6±0.11 points, the absolute survival rate after 12 hours reached to 91.2±1.90 c.u. and after 24 hours it was 177.6±2.68 c.u., while in the control variant these indicators, respectively, amounted to 7.1±0.23; 85.2±1.34 and 163.2±6.84.

The effectiveness of the studied vitamin can be due to the fact that vitamin E is the main natural antioxidant. Its functions are to protect cells from damaging reactions (peroxidation) resulting from a number of normal metabolic processes and from endo -, exogenous toxic products. The protective effect of vitamin E is mainly directed at biological membranes.

Vitamin E (tocopherol) is an important element of the antioxidant system: it prevents damage to cell walls by neutralizing hydrogen peroxide and other reactive oxygen species; it is necessary for the growth of new cells, for the normal functioning of the immune system.

Vitamin E intake reduces the “severity” of oxidative stress in testicular tissue, increases spermatozoa motility, and positively affects their ability to penetrate the egg (Рузаев, 2015).

Vitamin E is synergistic with retinol and selenium, i.e. by a simultaneous intake these substances exhibit marked efficacy at lower doses than when used separately, due to the mutual prevention of oxidation in the intestine and in tissues.

In the next series of experiments was used vitamin C (ascorbic acid) because it has an important role in human and animal life. Vitamin C exhibits antioxidant properties, participates in the regulation of carbohydrate metabolism and blood clotting, promotes tissue regeneration, increases the body's resistance to infections, reduces the human need for some vitamins (Фролова, 2009).

In addition, vitamin C being an antioxidant, it can inhibit the initiation of the process, stop the chain reaction, destroy hydroperoxides, and also have a neutralizing effect, i.e. it can act on almost all phases of lipid peroxidation, can act as a synergism of vitamin E (Узбеков, 2016).

Since ascorbic acid (vitamin C), together with formed from it the dehydroascorbic acid make up the redox system, which transfers hydrogen. This vitamin can participate in many biochemical processes of the cell. In this regard, vitamin C has been tested by us as an antioxidant in the medium for dilution and storage of boar semen. The results of the experiments are presented in Table 2.

Table 2. The effectiveness of the use of vitamin C in the composition of the medium for dilution of boar sperm

№	Concentration of vitamin, mg/ml	Motility after dilution, points	Absolute survival rate, conventional units	
			after 12 hours	after 24 hours
1	BM - control	5.7 ± 0.29	68.4 ± 3.42	112.6 ± 2.16
2	BM + 0.063	6.3 ± 0.14	74.4 ± 2.68	150.8 ± 3.21*
3	BM + 0.125	6.0 ± 0.01	72.0 ± 0.01	140.9 ± 2.68*
4	BM + 0.250	4.5 ± 0.28	58.8 ± 3.29	121.3 ± 4.24
5	BM + 0.500	4.0 ± 0.01	48.0 ± 0.01	90.2 ± 10.16
6	BM + 1.0	2.8 ± 0.22	31.2 ± 3.35	63.1 ± 9.18

* The differences are statistically authentic

The data of the Table 2 show that the best variant is a medium containing 0.063 mg/ml. Its use allows increasing the absolute survival rate of spermatozoa by a significant difference compared to the control variant. Further increase in the concentration of the test substance causes a decrease in motility and absolute survival rate of spermatozoa. The protective effect of ascorbic acid, both inside and outside the cell, can be explained by the recovery of oxygen free radicals in the presence of glutathione and alpha-tocopherol. When combined with vitamin A, B_6, iron ions and selenium, their effects are enhanced (Combs et al., 2012).

Due to the fact that folic acid is necessary for the formation of maintenance in a healthy state of new cells, as well as the normal formation of spermatozoa, it is advisable to study the effectiveness of this vitamin in the development of mediums for dilution and storage of sperm of farm animals. The results of our researches are presented in Table 3.

Analysis of the data presented in Table 3 allows us to note that the concentration of the tested drug is in the range of 0.025-0.4 mg/ml.

Table 3. The effectiveness of the use of vitamin B_9 in the composition of the medium for dilution of boar sperm

№	Concentration of vitamin, mg/ml	Motility after dilution, points	Absolute survival rate, conventional units	
			after 12 hours	after 24 hours
1	BM - control	6.5 ± 0.43	75.6 ± 2.85	144 ± 13.63
2	BM + 0.025	7.4 ± 0.27	86.4 ± 2.68	156 ± 10.39
3	BM + 0.05	7.4 ± 0.27	87.6 ± 2.68*	160.8 ± 21.47
4	BM + 0.1	7.3 ± 0.22	87.6 ± 2.68*	160.8 ± 21.47
5	BM + 0.2	6.8 ± 0.52	82.8 ± 4.90	144 ± 22.04
6	BM + 0.4	6.6 ± 0.48	70.8 ± 6.48	141 ± 17.18

* The differences are statistically authentic

Herewith the optimum is the 3rd variant with a vitamin concentration of 0.05 mg/ml. It is in this variant that a higher survival rate of spermatozoa is observed after 12 hours of sperm storage at 18-20°C, which is more on 14% than the experimental version. A similar trend is observed after 24 hours. The effectiveness of folic acid use may be due to the fact that in the presence of vitamin C, folic acid is converted into its main active form - tetrahydrofolic acid, the synthesis of which is carried out with the participation of the enzyme dehydrofolate reductase. In addition, folic acid and its derivatives have acceptor properties in relation to the hydrogen and are involved in the transfer of one-carbon groups, for example, methyl and formyl groups, from one organic compound to another, which determines its participation in redox processes (Владимиров, 1972). Another one feature has vitamin B_9. It refers to the compounds with conjugate bonds that predetermine the energy and thermodynamic stabilization of the system. This stability can give a variety of properties to synthetic mediums and cause an increase the resistance of spermatozoa to environmental factors. Together with vitamins B_{12}, C, B_6, B_2 and iron preparations exhibits increased activity.

Of particular interest is vitamin BT, one of the most important functions of which is the transmembrane transport of medium and long chain fatty acids in the mitochondria, where occurs their beta-oxidation to acetylcoenzyme A, which is a substrate for the formation of ATP in the carboxylic acid cycle (Harmeyer, 2002). Besides this L-carnitine performs several other functions such as: improving performance, accelerating growth, increasing strength and muscle mass, regulation of lipid metabolism, reducing cholesterol levels, antioxidant and antihypoxic effect, neuro-, hepato-and cardioprotective action, improve digestion, contribute to the normalization of the main metabolism, stimulates the nervous system, immunity and spermatogenesis. The main physiological function of L-carnitine and its acyl derivatives is the transfer of fatty acid residues from the cytoplasm to the mitochondrial matrix through the internal mitochondrial membrane. This is necessary for the formation of energy that is spent on the life support of the body's cells. By participating in the mitochondrial synthesis of ATP, L-carnitine and acetyl-L-carnitine are able to protect these organelles from oxidative stress by removing toxic acyl groups. The presence of an additional acyl group allows L-carnitine to more easily penetrate into the mitochondria and, as a result, more effectively perform its functions (Иванов et al., 2012).

In this regard, vitamin BT served as the object of studying the effectiveness of its use in the composition of the medium for the dilution of boar semen. The generalized results of such experiments are presented in Table 4.

Table 4. The effect of vitamin BT on the physiological parameters of diluted sperm of boars

№	Concentration of vitamin, mg/ml	Motility after dilution, points	Absolute survival rate, conventional units	
			after 12 hours	after 24 hours
1	BM - control	6.8 ± 0.42	74.7 ± 5.02	132.1 ± 18.00
2	BM + 0.02	7.6 ± 0.27	88.8 ± 5.37	165.6 ± 10.73
3	BM + 0.04	7.6 ± 0.27	91.2 ± 3.29*	177.6 ± 6.57*
4	BM + 0.08	7.6 ± 0.27	91.2 ± 3.29*	177.7 ± 6.57*
5	BM + 0.16	7.0 ± 0.35	84.0 ± 4.24	160.8 ± 9.10
6	BM + 0.32	6.9 ± 0.27	81.6 ± 4.03	160.8 ± 9.10

* The differences are statistically authentic

From the data of table 4 it follows that 0.08 mg/ml is the optimal concentration of vitamin BT, the use of which allows to increase the absolute survival rate of spermatozoa after 12

hours of storage by 22%, and after 24 hours - by 35% compared with the control variant. The positive effect of carnitine, apparently, is largely due to its ability to form complexes with various organic compounds, which are intermediate products of oxidative processes. These substances accumulating in the cell have a membrane-toxic effect and inhibit the activity of a number of enzymes. Removal of toxins from cells is made in the form of acylcarnitines. The positive effect of the studied vitamins may be due to their participation in redox reactions. However, might work and another mechanism. Therefore, in the next series of experiments was determined the amount of malonic dialdehyde, which is one of the main products of lipid peroxidation (Table 5).

Table 5. The effect of vitamins on the content of malonic dialdehyde in boar semen

№	Experience variants	Content of vitamin, mg/ml	Content of malonic dialdehyde, nm/billion		Malonic dialdehyde,%
			after dilution	after 24 hours	
1.	BM + vitamin E (alpha-tocopherol acetate)	0.5	12.8 ± 0.86	28.6 ± 1.87*	123.4
2.	BM + vitamin C (ascorbic acid)	0.06	12.8 ± 0.86	35.3 ± 2.63	175.8
3.	BM + vitamin B_9 (folic acid)	0.05	12.8 ± 0.86	29.1 ± 2.11*	127.3
4.	BM + vitamin BT (carnitine)	0.08	12.8 ± 0.86	30.6 ± 3.16*	154.3
5.	BM, without vitamins, control	0	12.8 ± 0.86	41.3 ± 3.51	222.6

* The differences are statistically authentic compared with the control variant

From Table 5 it follows that vitamins E, C, B_9 and BT have antioxidant properties, as evidenced by the lower rates of accumulation of malonic dialdehyde in boar semen in the case of its storage at 18-20°C.

CONCLUSIONS

Vitamins E, C, B_9 and BT in addition to known properties, also have specific antioxidant features, as evidenced by the different rates of accumulation of malonic dialdehyde in the process of dilution and storage of boar semen.

Whether all vitamins have antioxidant properties is to be studied in perspective using a wider and new arsenal of research methods.

Antioxidants, and in particular vitamins, never completely block the formation of lipid peroxidation products, they only slow down this process.

There is an inversely proportional relationship between the absolute survival rate of gametes and the malonic dialdehyde content.

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