

## THE IMPACT OF ADDITION OF REDUCED GLUTATHIONE AND BOVINE SERUM ALBUMIN TO THE EXTENDER ON THE QUALITATIVE INDEXES OF THE FROZEN–THAWED RAM SPERM

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**Abstract.** The study revealed the optimal concentration of reduced glutathione and bovine serum albumin in the lactose-yolk-tris-citrate-glycerine diluent in ram–sires sperm cryopreservation. It was shown that the addition of 5  $\mu\text{M}$  of reduced glutathione and 15  $\text{mg} \cdot \text{ml}^{-1}$  of bovine serum albumin into the lactose-yolk-tris-citrate-glycerine diluent contributes to the increase in frozen–thawed sperm activity by 11.0%, its resistance to cryopreservation by 6.0%, intact acrosome by 9.5%, sperm surviving rate by 23.9%, absolute survival indicator by 21.8%, sperm kinetic indicators: VCL – by 14.3%, VAP – by 12.5%, VSL – by 15.4%, LIN – by 0.4%, STR – by 2.2%, as well as fertilization rate by 12.0%. These results prove the positive effect of the addition of bioactive substances into the diluent on the qualitative features of frozen–thawed sperm.

**Key words:** bovine serum albumin, ewes, frozen–thawed sperm, ram–sires, reduced glutathione, sperm kinetic indicators

### INTRODUCTION

Application of artificial insemination is an important way of improving the reproduction in sheep, while it improves livestock, creates new high–producing

breeds, intraspecific types and lines. One of the relevant factors in artificial insemination efficiency is the improvement of the cryopreservation method and the storage of deep-frozen sperm, which supports the efficacy of the most valuable ram-sires usage, regardless of the season, distance and climate conditions [Holt 2000, Salamon and Maxwell 2000].

The effectiveness of sperm cryopreservation largely depends on the composition of synthetic diluents. The purpose of the diluents is to provide the appropriate protection of sperm from the adverse factors during cryopreservation, caused by low and ultralow temperature effects [Choudhry et al. 1995, Anghel and Zamfirescu 2010]. Lactose, yolk and glycerin are used as major components of sperm diluents in order to preserve acrosome. To prevent excessive accumulation of toxic products of lipid peroxidation in the ram sperm, it is proposed to use different antioxidants in the composition of diluents in the process of equilibration and storage of cooled and deep-frozen sperm [Epishina 2009]. It is established that using antioxidants, which have protective properties, in the diluents while freezing the ram sperm significantly increases the effectiveness of insemination [Kheradmand et al. 2006, Deryazhentsev and Epishina 2008].

In the literature, bovine serum albumin (BSA) and thiol compounds are reported to have a positive impact in retaining of biological values in the animal sperm. A number of authors indicate that the macromolecule of BSA has a protective effect on semen enzymes. The use of BSA as part of the diluent for ram sperm, stored at the temperature of 16°C, increases the motility and survival of sperm [Uysal and Bucak 2007].

Taking into account the aforementioned, we have studied the impact of reduced glutathione and bovine serum albumin in the lactose-yolk-tris-citrate-glycerine diluent (LYTCGD) in freezing the sperm of Ukrainian Carpathian Mountain breed rams on the following parameters: frozen-thawed sperm motility, surviving rate, acrosome integrity, biochemical parameters and fertilization rate.

## MATERIAL AND METHODS

Studies on the impact of reduced glutathione and bovine serum albumin as part of lactose-yolk-tris-citrate-glycerine diluent on the sperm qualitative features and its fertilizing capacity were conducted on the rams and ewes of the Ukrainian Carpathian Mountain breed from the smallholding farm organization “Saldobosh” in Khust district, Zakarpattia region.

The obtained ejaculate with the activity of 80–90% was diluted 1:3 with lactose-yolk-tris-citrate-glycerine diluent with 3, 5, 10 and 20 µM concentrations of glutathione. In the control group the sperm was diluted with the same diluent without reduced glutathione. After that, using the Minitube equipment, 0.25 ml

straws were filled with the diluted sperm, put into the refrigerator at the temperature of 4°C, equilibrated for 3 hours and then held above nitrogen vapour for 20 minutes (-120°C) in a special vessel. Then, the straws were packaged in tapes and dipped in liquid nitrogen for storage.

Similarly to the studies with the reduced glutathione, we studied the impact of a different content of bovine serum albumin (5, 10, 15 and 20 mg · ml<sup>-1</sup>) in lactose-yolk-tris-citrate-glycerine diluent on the aforementioned qualitative characteristics of ram-sires sperm.

The activity of sperm after equilibration and cryopreservation, sperm kinetic indicators, sperm resistance to freezing, sperm surviving and absolute survival indicator were determined in the frozen-thawed sperm. Sperm resistance to freezing was determined in percentage terms of the number of active gametes with a straight-forward movement after thawing to the number of sperm with a straight-forward movement after equilibration. Thawed sperm survival rate was determined under the microscope (×200) at 38°C every 0.5 hour until no motile spermatozoa were observed. The sperm absolute survival indicator at +38°C was calculated using a simplified formula:  $Sa = a^1/2 + \sum(a \cdot t) \cdot n$ , where *Sa* – absolute survival indicator; *a* – assessment of sperm motility in examination points; *t* – indicator of time interval between the previous and the next test of sperm; *n* – number of observations.

To compare the fertilizing ability of the sperm frozen in LYTCGD (control) and the same diluent improved with the content of reduced glutathione and BSA (experimental), artificial insemination of ewes was conducted.

For artificial insemination, clinically healthy sheep of average bodyweight, with evident signs of heating and selected by a ram-probe, were chosen to be inseminated by a laparoscopic method. A total of 99 females were inseminated, including 52 ones being inseminated with the sperm frozen in the control diluent and 47 ewes – in the improved one.

All calculations were performed using Statistica software [StatSoft 2009].

## **RESULTS AND DISCUSSION**

The studies revealed that the highest indexes of frozen-thawed sperm activity, sperm kinetic indicators, sperm resistance to freezing, acrosome preservation and surviving were observed in the diluent with the 5 µM concentration of reduced glutathione and the 15 mg/ml content of BSA.

These results indicate that the lactose-yolk-tris-citrate-glycerine diluent can be improved by adding 5 µM of reduced glutathione and 15 mg · ml<sup>-1</sup> of BSA. To confirm this hypothesis we carried out the comparative evaluation of qualita-

tive indicators of ram–sires sperm in the improved environment with the control LYTCGD (Table 1).

Table 1. The impact of glutathione and BSA as part of the environment for freezing the ram sperm on the sperm qualitative characteristics,  $M \pm m$ ,  $n = 10$

Tabela 1. Wpływ glutationu i BSA jako składnika środowiska do zamrażania nasienia tryków na cechy jakościowe plemników,  $M \pm m$ ,  $n = 10$

| Indicators<br>Wskaźniki   | Diluent – Rozcieńczalnik                |  |
|---|---|--|
|   | Control (LYTCG)<br>Kontrolna<br>(LYTCG) | Experimental (LYTCG + 5 $\mu$ M glutathione + 15 mg $\cdot$ ml <sup>-1</sup> BSA)<br>Doświadczalna (LYTCG + 5 $\mu$ M glutathion + 15 mg $\cdot$ ml <sup>-1</sup> BSA) |
| Motility of collected semen, %<br>Ruchliwość pobranego nasienia, %                  | 90.15 $\pm$ 1.65                        | 90.15 $\pm$ 1.65   |
| Motility of semen after equilibration, %<br>Ruchliwość nasienia po równoważeniu, %  | 73.82 $\pm$ 2.35                        | 84.46 $\pm$ 1.52**   |
| Motility of frozen–thawed semen, %<br>Ruchliwość mrożonego–roztopionego nasienia, % | 41.24 $\pm$ 1.31                        | 52.26 $\pm$ 2.13***  |
| Sperm resistance to freezing, %<br>Odporność plemników na zamrażanie, %             | 55.86 $\pm$ 3.41                        | 61.87 $\pm$ 2.36   |
| Sperm with damaged acrosome, %<br>Plemniki z uszkodzonym akrosomem, %               | 27.50 $\pm$ 2.85                        | 18.00 $\pm$ 1.65*  |
| Sperm surviving rate, hours<br>Współczynnik przeżywalności plemników, godziny       | 7.10 $\pm$ 0.48                         | 8.80 $\pm$ 0.47*   |
| Absolute survival indicator, S.U<br>Całkowity wskaźnik przeżywalności, S.U          | 13.24 $\pm$ 0.96                        | 16.12 $\pm$ 1.15*  |

Values differ significantly at: \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ .

Wartości różnią się istotnie przy poziomach istotności: \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ .

These data indicate that there is a positive impact of a combined use of reduced glutathione and BSA in LYTCGD in the reported concentrations on the qualitative characteristics of frozen–thawed sperm that were investigated. The integrated addition of reduced glutathione and BSA in the experimental diluent improved the quality of sperm after equilibration on average by 10.6%.

The activity of frozen–thawed sperm in the improved diluent accounted for 52.26  $\pm$  2.13% and was higher than the control diluent by 11.0%. The addition of reduced glutathione and BSA improved the sperm resistance to freezing by 6.0% and acrosome preservation by 9.5%. The sperm surviving in the improved diluent accounted for 8.80  $\pm$  0.47 h, which is by 23.9% higher than in the control one. The sperm absolute survival index was also higher in the improved diluent compared to the control by 21.8%.

The sperm kinetic parameters also differed significantly between the control and experimental (improved) diluent (Table 2).

Table 2. The impact of glutathione and BSA as part of the environment for ram sperm cryopreservation on frozen-thawed sperm kinetic parameters,  $M \pm m$ ,  $n = 10$ Tabela 2. Wpływ glutationu i BSA jako składnika środowiska do krioprezerwacji nasienia tryków na parametry kinetyczne plemników po mrożeniu,  $M \pm m$ ,  $n = 10$ 

| Indicators<br>Wskaźniki   | Diluent – Rozcieńczalnik                |   |
|---|---|---|
|   | Control (LYTCG)<br>Kontrolna<br>(LYTCG) | Experimental (LYTCG + 5 $\mu$ M glutathione + 15 mg $\cdot$ ml <sup>-1</sup> BSA)<br>Doświadczalna (LYTCG + 5 $\mu$ M glutathione + 15 mg $\cdot$ ml <sup>-1</sup> BSA) |
| Motility of frozen-thawed semen, %<br>Ruchliwość mrożonego nasienia, %    | 58.36 $\pm$ 3.10                        | 59.18 $\pm$ 2.89  |
| Sperm with straight-forward movement, %<br>Plemniki o ruchu postępowym, % | 41.24 $\pm$ 2.46                        | 52.26 $\pm$ 2.44**  |
| VCL, $\mu$ m $\cdot$ s <sup>-1</sup>                                      | 169.45 $\pm$ 3.63                       | 193.65 $\pm$ 2.19***  |
| VAP, $\mu$ m $\cdot$ s <sup>-1</sup>                                      | 88.12 $\pm$ 3.22                        | 99.16 $\pm$ 2.94*   |
| VSL, $\mu$ m $\cdot$ s <sup>-1</sup>                                      | 75.68 $\pm$ 3.16                        | 87.32 $\pm$ 2.85*   |
| LIN, %  | 44.66 $\pm$ 1.71                        | 45.09 $\pm$ 1.66  |
| STR, %  | 85.88 $\pm$ 3.22                        | 88.05 $\pm$ 3.61  |
| WOB, %  | 52.00 $\pm$ 1.64                        | 51.20 $\pm$ 1.48  |

Values differ significantly in: \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ .

Wartości różnią się istotnie przy poziomach istotności: \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ .

The indexes of curvilinear velocity (VCL), average path velocity (VAP) of a sperm head along its average trajectory, straight-line velocity of a sperm head along a straight line between the first and last positions of the trajectory (VSL) were higher in the improved diluent by 14.3, 12.5 and 15.4%, respectively in comparison to the control. Higher velocity indexes of the sperm in the improved diluent increased the degree of linearity in comparison to the control diluent by 0.4% and the degree of sperm straightness by 2.2%.

Thus, the introduction of 5  $\mu$ M of reduced glutathione and 15 mg/ml of bovine serum albumin into the lactose-yolk-tris-citrate-glycerine diluent contributed to the increase the qualitative indicators of ram-sires thawed sperm. The results of our studies are consistent with the data of Uysal [2007] and Nikolovski [2014], reporting the improved qualitative indexes of the frozen-thawed ram sperm after adding reduced glutathione to the freezing extender. Also Matsuoka et al. [2006] reported the increase in sperm activity, sperm survival and a decrease in sperm pathological forms after adding the BSA to the cryoconservation extender for ram sperm.

In order to ascertain the biological value of sperm frozen in the improved lactose-yolk-tris-citrate-glycerine diluent we have conducted experiments of the artificial insemination of the ewes from the smallholding farm organization “Saldobosh”. The results of this prove that the proposed improved diluent increases the

biological value of sperm and their fertilizing capacity (Table 3). In particular, the fertility of ewes inseminated with frozen–thawed sperm frozen in the improved diluent was higher by 12.0% in comparison with the animals inseminated with the frozen sperm using the control diluent.

Table 3. The impact of the improved environment of ram-sires sperm on the reproductive characteristics of ewes

Tabela 3. Wpływ poprawy środowiska mrożenia nasienia tryków na zdolność reprodukcyjną owiec

| Indicators<br>Wskaźniki   | Diluent – Rozcieńczalnik             |   |
|---|--------------------------------------|---|
|   | Control (LYTCG)<br>Kontrolna (LYTCG) | Experimental (LYTCG + 5 $\mu$ M<br>glutathione + 15 mg · ml <sup>-1</sup> BSA)<br>Doświadczalna (LYTCG + 5 $\mu$ M<br>glutathione + 15 mg · ml <sup>-1</sup> BSA) |
| Number of inseminated ewes<br>Liczba inseminowanych owiec   | 52                                   | 47  |
| Fertilization rate of the ewes after first<br>insemination, %<br>Współczynnik zapłodnienia owiec po pierwszej<br>inseminacji, %   | 37.0–71.1                            | 39.0–83.0   |
| Fertilization rate of the ewes after all<br>inseminations, %<br>Współczynnik zapłodnienia owiec we wszystkich<br>inseminacjach, % | 94.2                                 | 97.9  |
| Number of ewes that have lambed<br>Liczba macierek wykończonych   | 49                                   | 46  |
| Number of lambs lambed<br>Liczba urodzonych jagniąt   | 55                                   | 51  |
| Fertility index, %<br>Wskaźnik płodności, %   | 105.8                                | 108.5   |
| Number of lambs weaned<br>Liczba jagniąt po odsadzeniu  | 51                                   | 48  |
| Fertility index after weaning, %<br>Wskaźnik płodności po odsadzeniu, %   | 98.1                                 | 102.1   |
| Lamb survival, %<br>Odsetek przeżywalności jagniąt, %   | 92.7                                 | 94.1  |

In particular, the rate of fertilization of ewes after first insemination with thawed sperm frozen in the improved diluent was higher by 12.0% in comparison with the animals, inseminated with the frozen sperm using the control diluent. The rate of fertilization from all inseminations was also higher by 3.7 percent from the insemination with the sperm frozen in the improved diluent, and accounted 97.9%, which had a positive impact on the fertility of ewes, at 108.5% at the birth of lambs. The lamb survival was also higher by 1.4 percent in the group of the ewes inseminated with deep–frozen sperm in the improved diluent.

## CONCLUSION

A positive impact of reduced glutathione and bovine serum albumin on activity, surviving and fertilizing capacity of ram-sires frozen-thawed sperm is caused by their antioxidant effects on membrane structures of the sperm. The obtained results prove that the use of the improved environment for deep freezing of ram-sires sperm is effective.

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## WPLYW DODATKU ZREDUKOWANEGO GLUTATIONU I ALBUMINY SUROWICY BYDŁĘCEJ DO ROZCIEŃCZALNIKA NA JAKOŚĆ MROŻONEGO NASIENIA TRYKA

**Streszczenie.** W doświadczeniu wykazano optymalne stężenie dodatku zredukowanego glutationu i albuminy surowicy bydłowej do rozcieńczalnika zawierającego laktozę, żółtko jaja kurzego, tris, cytrynian i glicerynę, wykorzystywanego do mrożenia nasienia tryków. Wykazano, że 5  $\mu\text{M}$  zredukowanego glutationu i 15  $\text{mg} \cdot \text{ml}^{-1}$  albuminy surowicy bydłowej do badanego rozcieńczalnika zwiększa aktywność mrożonych plemników o 11,0%, ich odporność na krioprezerwację o 6,0%, integralność akrosomu o 9,5%, współczynnik przeżywalności o 23,0% i całkowity wskaźnik przeżywalności o 21,8%. Wykazano także poprawę następujących parametrów ruchu plemników: VCL – o 14,3%, VAP – o 12,5%, VSL – o 15,4%, LIN – o 0,4%, STR – o 2,2% oraz współczynnika zapłodnialności o 12%. Powyższe wyniki dowodzą pozytywnego wpływu dodatków substancji bioaktywnych do rozcieńczalnika na cechy jakościowe mrożonego nasienia.

**Słowa kluczowe:** albumina surowicy bydłowej, kinetyczne parametry plemników, mrożone nasienie, owce, tryki, zredukowany glutation

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