POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 28(3): 379–384, Y. 2013

INFLUENCE OF ANTIOXIDANTS ON SPERMATOZOA IN THE SHORT-TERM STORAGE OF SALMONIDAE MILT*

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K e y w o r d s: rainbow trout, Arctic chair, milt, motility, CASA.

A b s t r a c t

Short-term storage of semen in cooling conditions (+4°C) is one way to perfect artificial fish reproduction. In this experiment, we attempted to add antioxidants (vitamins C and E, glutathione and cysteine) during the storage of Arctic char (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) semen. An analysis of the CASA parameters showed that addition of the antioxidants to semen during storage did not benefit spermatozoa motility of studied salmonids. An analysis of the parameters showed that added vitamins C and E did not influence the sperm motility of Salmonidae during semen storage. The addition of glutathione and cysteine significantly worsened the vitality of Arctic char and rainbow trout sperm.

WPŁYW DODATKU ANTYOKSYDANTÓW NA KRÓTKOOKRESOWE PRZECHOWYWANIE NASIENIA RYB ŁOSOSIOWATYCH

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S ł o w a k l u c z o w e: pstrąg tęczowy, palia alpejska, mlecz, ruchliwość, CASA.

A b s t r a k t

Krótkookresowe przechowywanie nasienia w warunkach chłodniczych (+4°C) jest jednym ze sposobów doskonalenia metod sztucznego rozrodu u ryb. W pracy podjęto próbę zastosowania dodatku antyoksydantów (witaminy C, witaminy E, glutationu i cysteiny) w trakcie przechowywania

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* The study was supported by National Science Centre Nr N 311 515 640.

nasienia palii alpejskiej (*Salvelinus alpinus*) oraz pstrąga tęczowego (*Oncorhynchus mykiss*). W analizie parametrów ruchu plemników wykazano, że dodatek witaminy C oraz witaminy E nie wpływa istotnie na ruchliwość plemników ryb łososiowatych w trakcie przechowywania nasienia. Dodatek glutationu i cysteiny w istotnym stopniu pogorszył żywotność plemników palii alpejskiej i pstrąga tęczowego.

Introduction

In recent years, the possibility of short- and long-term sperm storage in fish reproduction has become more and more important. The formation of free oxygen radicals, which occurs during cryopreservation and semen storage in chilling conditions (+4°C), is responsible for decreased stability of the cell membrane, impaired functioning of the mitochondria and DNA fragmentation. These processes influence the lowering of sperm motility parameters, and thus as a result decrease in sperm fertility (BILLARD et al. 2004, SANOCKA and KURPISZ 2004, LAHNSTEINER et al. 2011).

In natural conditions, seminal plasma protects sperm from free oxygen radicals. Semen storage in chilling conditions however must be diluted with various buffers, which demonstrates the insufficiency of seminal plasma's protective role. Experiments by BUCAK et al. (2007) and THUWANUT et al. (2008) showed that supplementing antioxidants to buffers used during cryopreservation aids prevention of sperm damage due to the presence of free radicals. The positive effect of enriching fish feed with antioxidants such as vitamins C and E on fertility has been known for a while (CIERESZKO and DABROWSKI 1995 – *Oncorhynchus mykiss*; MANSOUR et al. 2006 – *Salvelinus alpinus*; METWALLY and FOUAD 2009 – *Ctenopharyngodon idellus*).

Results by GLOGOWSKI et al. (2008) demonstrated the detrimental effect of oxygen on the survival of rainbow trout sperm stored *in vitro*. The addition of albumin, a protein with antioxidant properties, significantly lengthened the period of trout semen storage (*Oncorhynchus mykiss*) (KOWALSKI et al. 2009). In this paper, we attempted to improve the buffer composition for short-term storage of Arctic char (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) semen, by the addition of selected antioxidants to the extender solution, such as vitamins A and E, glutathione and cysteine.

Material and Methods

Semen from Arctic char (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) were obtained during the autumn mating season in 2011 from the Pstrąg Tarnowo Fish Hatchery (Wielkopolski Voivodeship). The fish were put

under anesthesia and then semen was gathered through abdominal massage. The semen from males ($n=5$) was mixed with Morisawa's buffer with 1% albumin at a proportion 1:10, the control sample. In order to determine antioxidants' influence on semen storage, we used Morisawa's buffer, composed of 0.1 M NaCl, 0.04 M KCl, 0.03 M $CaCl_2$, 0.0015 M $MgCl_2x6H_2O$, 0.05 M Tris, 0.5% albumin, pH 8.5 (MORISAWA and MORISAWA 1988) with a supplement of 1 mM of vitamin C, 1.5 mg ml^{-1} of vitamin E, 5 mM of glutathione and 5 mM of cysteine. After two days of storage in chilling conditions (+4°C), an antibiotic was added to every sample (penicillin and streptomycin in proportions of 100 U ml^{-1} and 100 μg ml^{-1}, respectively). The samples were mixed every 24 h to avoid the negative effects of sperm sedimentation. In the prepared samples, the sperm parameters were marked every two or three days with the CASA system. Motility activating fluids were used for the activation of sperm movement, Billard fluid with an addition of 0.5% albumin (BILLARD 1985). Activation was done in the small eppendorf tube where sperm samples were mixed with activation solutions supplemented. 0.5 μl of sperm samples were mixed with 200 μl of activation solution. After activation 1 μl of each sample was transferred to the 12 wells Teflon coated slide glass (Tekdon INC. 40521 State Road 64 Myakka City, Florida 34251). Motility analysis was carried out using the Crismas equipment (Image House CRISMAS Company Ltd.). Sperm movement was documented 6 second after activation with a Basler 202K digital camera integrated with an Olympus BX51 microscope. Total sperm velocity, VCL (μm s^{-1}), and the percentage of motile sperm, MOT (%) were analysed.

The statistical analysis was made using the GraphPad Prism program (GraphPad Software Inc., USA), incorporating the ANOVA two-way analysis of variance. The differences between particular test subjects were established by the Bonferroni post-test.

Results

Among the four applied antioxidants, adding vitamins C and E to the semen of Arctic char brought the best results, as well as control samples, diluted in Morisawa buffer without any additions (Table 1). The other two antioxidants, glutathione and cysteine, caused a complete loss of movement after ten days of storage, whereas motile sperm was still observed in the control sample and the samples with vitamins C and E after fifteen days of storage. Rainbow trout semen diluted with Morisawa buffer (control) or with added vitamins C and E maintained motility longer than Arctic char semen. After eighteen days of storage at +4°C, 28–35% of trout sperm was observed to

be motile (Table 1). The motile spermatozoa characterized the similar VCL values for every day of experiment.

Table 1
Arcic char and rainbow trout sperm motility parameters during 15 or 18 days of storage at +4°C

Time (day)	Control	Vit. C 5 mM	Vit. E 1.5 mg ml^{-1}	Glutathione 5 mM	Cysteine 5 mM
Arctic char MOT [%]					
1	89^{ax}	72^{ax}	87^{ax}	69^{ax}	84^{ax}
3	91^{ax}	48^{bx}	87^{ax}	45^{bxy}	34^{by}
5	83^{ax}	46^{bxy}	65^{abx}	24^{cy}	23^{cy}
8	64^{axy}	26^{byz}	29^{by}	21^{by}	14^{by}
10	43^{ayz}	30^{abyz}	42^{ay}	14^{by}	11^{byz}
12	35^{ayz}	21^{ayz}	17^{ay}	0^{bz}	0^{bz}
15	12^{az}	7^{az}	14^{ay}	0^{az}	0^{az}
VCL [μm s^{-1}]					
1	271^{ax}	272^{ax}	280^{ax}	255^{ax}	255^{ax}
3	294^{ax}	261^{ax}	300^{ax}	246^{ax}	291^{ax}
5	300^{ax}	172^{ax}	231^{ax}	187^{ax}	172^{ax}
8	295^{ax}	147^{ax}	154^{ax}	154^{ac}	143^{axy}
10	271^{ax}	164^{ax}	217^{ax}	128^{abx}	69^{by}
13	229^{axy}	141^{axy}	142^{ax}	0^{by}	0^{bz}
15	91^{ay}	108^{ay}	134^{ax}	0^{by}	0^{bz}
Rainbow trout MOT [%]					
1	96^{ax}	93^{ax}	88^{ax}	78^{ax}	78^{ax}
3	94^{ax}	86^{axy}	84^{ax}	55^{bx}	32^{by}
5	84^{ax}	68^{ay}	89^{ax}	31^{by}	50^{bz}
8	77^{axy}	58^{ayz}	85^{ax}	12^{bz}	23^{bq}
10	65^{ay}	56^{ayz}	75^{ax}	4^{bz}	18^{bq}
12	62^{ayz}	48^{az}	68^{axy}	0^{bz}	10^{bq}
15	51^{az}	38^{az}	53^{ay}	0^{bz}	0^{bq}
18	35^{az}	28^{az}	29^{ay}	0^{bz}	0^{bq}
VCL [μm s^{-1}]					
1	244^{ax}	261^{ax}	251^{ax}	262^{ax}	288^{ax}
3	308^{ax}	289^{ax}	280^{ax}	263^{ax}	231^{ax}
5	280^{ax}	268^{ax}	292^{ax}	175^{bx}	233^{bx}
8	270^{ax}	253^{ax}	290^{ax}	66^{by}	216^{xy}
10	285^{ax}	244^{ax}	289^{ax}	20^{byz}	170^{cy}
12	273^{ax}	233^{ax}	250^{ax}	0^{bz}	89^{by}
15	227^{ax}	177^{ax}	234^{ax}	0^{bz}	0^{bz}
18	234^{ax}	164^{ax}	241^{ax}	0^{bz}	0^{bz}

Control sample was diluted 1:9 with Morisawa buffer (Morisawa and Morisawa 1988). Vit. C – diluted samples with vitamin C addition, vit. E – diluted samples with vitamin E addition, glutathione – diluted samples with glutathione addition, Cysteine – diluted samples with cysteine addition. Data shows the percentage of motile spermatozoa (MOT) and curvilinear velocity (VCL). Data represent mean values (n=5). Different letters (*a, b, c*) indicate statistically significant differences between the buffers at the same time; the letters *x, y, z, q* indicate statistically significant differences between time points for each buffer ($p \leq 0.01$).

Discussion

Our previous experiments on antioxidant supplements' effect on sperm survival of ide (*Leuciscus leuciscus*) after cryopreservation demonstrated that supplements of vitamins C and E as well as glutathione and cysteine improve some of the sperm motility parameters (SAROSIEK et al. 2011):

VCL – curvilinear velocity of sperm;

VSL – straight-line velocity;

VAP – average path velocity of sperm (SAROSIEK et al. 2011). We also concluded that a supplement of antioxidants improves the condition of sperm after freezing and increases the percentage of ide eggs fertilization by 20% in comparison with the fertility of frozen sperm without antioxidants. However, contrary to cryopreservation results, the antioxidants supplementation did not significantly lengthen the period of short-term storage for salmonid semen compared to the control sample.

In mammalians several negative effects of reactive oxygen species on sperm viability have been recorded. They may cause lipid peroxidation of sperm cell membranes, damage of midpiece structure or loss of motility and infertility (SIKKA et al. 1995, SIKKA 2004). KOWALSKI et al. (2009) observed shortened time of Rainbow trout spermatozoa viability during storage at +4°C in oxygen atmosphere. On the other hand, high concentration of uric acid (strong antioxidant) in fish seminal plasma have been related to protection of spermatozoa against oxygen free radicals (CIERESZKO et al. 1999).

Our latest experiment showed (SAROSIEK et al. 2013), that a supplement of antioxidants (vitamins C and E, glutathione and cysteine) during the storage of perch semen (*Perca fluviatilis*) did not benefit its vitality. The results on perch and salmonid semen indicated that the semen dilution with immobilization buffer was sufficient for short-term storage. The antioxidants addition did not improve the sperm motility parameters. It should be investigated wheatear it might be a cause of high antioxidant potential of their seminal plasma (SŁOWIŃSKA et al. 2013) and therefore any further supplementation did not change overall effectiveness of protection against free radicals.

Translated by JEEF TAYLOR

Accepted for print 9.10.2013

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