Chemical induction of haploid gynogenesis in sterlet *Acipenser ruthenus*

I. Lebeda, I. Gazo, M. Flajshans

South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic

ABSTRACT: Chromosomal manipulations in sturgeons, particularly gynogenesis, are interesting due to the potential to change female ratio in progeny that can be useful for caviar production. The optimization of UV treatment for induction of gynogenesis is complicated due to high and variable optical density of the milt due to differential spermatozoa concentration, and because of sensitivity of spermatozoa's motility apparatus. Therefore in this study we compared chemical methods of sperm treatment as an alternative to short wave-length UV treatment; evaluation considers impact on spermatozoa motility, DNA integrity, and efficiency of DNA inactivation. Dimethyl sulfate (DMS) in concentrations of 2.5–30mM was applied to spermatozoa in order to inactivate DNA. Also ethidium bromide (EB), psoralen (PS), and 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) were used to increase sensitivity of spermatozoa's DNA to long wavelength UV-A light (360 nm). CASA analyses of treated sperm showed strong negative effects on spermatozoa motility with the increasing concentration of active substances. Additionally in case of PS, EB, and DMS treatment comet assay did not reveal significant DNA damage of sperm at the range of concentrations relatively safe for spermatozoa motility. Flow cytometric analysis of relative DNA content in larvae resulting from activation of normal ova of sterlet with the treated sperm showed low efficiency of haploid gynogenesis induction. The putative gynogenetic larvae were found after treatment with PS in concentrations higher than 18μM and EB higher than 10μM followed by UV-A irradiation at the dose of 900 J/m^2 and DMS up to 5mM. Because of an overwhelming impact on the sperm motility and relatively low DNA damage, treatment of sperm with PS, EB or DMS did not prove efficient compared with a widely used UV-C irradiation treatment. In contrast, treatment with AMT followed by UV-A showed lower influence on spermatozoa motility and higher efficiency of DNA damaging resulting in the higher percentage of gynogenotes in the progeny, thus could be considered as a possible substitution for UV-C treatment.

Keywords*:* sturgeons; chromosomal manipulation; dimethyl sulfate; psoralen; comet assay

INTRODUCTION

Sturgeons are one of the oldest fish families including the genera *Acipenser*, *Huso*, *Scaphirhynchus,* and *Pseudoscaphirhynchus*. The number of research and developmental studies on sturgeon biology and biotechnology is constantly increasing because their survival in the wild is seriously endangered (Fontana et al. 2001) and they are highly prized for their caviar and flesh. The worldwide population of sturgeons is constantly declining due to over-fishing, water pollution, and habitat degradation (Billard and Lecointre 2001). It is anticipated that the future of sturgeon caviar and flesh production is in developing the aquaculture biotechnologies. Thus it is highly demanding to elaborate methods of sex identification and manipulation with progeny sex ratio. Chromosomal

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (Projects "CENAKVA" No. CZ.1.05/2.1.00/01.0024 and "CENAKVA II" No. LO1205 under the NPU I program), by the Grant Agency of the University of South Bohemia in Česke Budějovice (GAJU) (Projects No. 086/2013/Z and No. 114/2013/Z), and by the Czech Science Foundation (GAČR) (Project No. 14-02940S).

manipulation methods, particularly gynogenesis, can be used for changing the female ratio in sturgeon progeny. Despite quite contradictory results of sturgeon sex determination system assessment, female heterogamety is commonly accepted (Keyvanshookoh and Gharaei 2010). In accordance with this hypothesis, high percentage of females in gynogenetic progeny was shown in a number of sturgeon species: *Acipenser transmontanus* 82% (Van Eenennaam 1999), *Acipenser baerii* 81% (Fopp-Bayat 2010), *Polyodon spathula* 80% (Shelton and Mims 2012), bester 70–80% (Omoto et al. 2005), *Acipenser brevirostrum* 65% (Flynn et al. 2006). Furthermore, the other applications of gynogenesis are related to the interesting ploidy level system of sturgeon and its evolution. Due to high homozygosity of gynogenetic progeny, it can be useful in genetic research and selection.

The processes of gynogenesis induction consist of two main steps – the inactivation of paternal DNA in sperm and the restoration of zygote's diploidy. Inactivation of genetic information in spermatozoa for activation of eggs is a critical step for gynogenetic induction protocol. The second step is quite similar to a triploidization method and represents an application of a shock to induce retention of the 2nd meiotic polar body (Fopp-Bayat et al. 2007). The most common way to inactivate DNA in spermatozoa is to irradiate them with UV, but a study on the influence of UV irradiation on motility of sturgeon spermatozoa showed high sensitivity of the motility apparatus to UV light (Recoubratsky et al. 2003; Dietrich et al. 2005). Furthermore, optimization of the UV treatment is complicated due to high optical density of sperm and significant difference in sperm density among individual males (Christensen and Tiersch 1994; Mims and Shelton 1998; Linhart et al. 2000).

The other way to induce gynogenesis is inactivation of DNA in spermatozoa by chemical agent that selectively damages DNA and provides minimum damage to spermatozoa's motility apparatus and acrosome. Tsoi (1969, 1974) used dimethyl sulfate (DMS) as chemical agent inactivating rainbow trout (*Oncorhynchus mykiss*) and peled (*Coregonus peled*) spermatozoa's chromosomal apparatus. He found similarity of survival rate dependence in eggs activated by DMS and UV treated spermatozoa. Increasing of concentrations above the lethal dose has led to the appearance of normally looking larvae. Inactivating ability of DMS was proved, after spermatozoa treating with high concentrations of DMS, by direct counting of chromosomes in obtained embryonic cells. Though eggs of peled showed quite low survival rate after insemination by sperm treated with DMS in the concentration of 7.7mM. Only 1.3% of embryos were hatched and only 0.16% of them reached the age of one month.

Various fluorescent DNA dyes have mutagenic activity (Matselyukh et al. 2005). For instance ethidium bromide (EB) can intercalate to DNA structure, and form covalent bonds under UV-light (Waring 1965; Cariello et al. 1988). Uwa (1965) obtained gynogenotes of medaka (*Oryzias latipes*) after treatment of sperm with toluidine blue followed by UV irradiation, although substantial part of embryos showed abnormalities and delay in development.

A widely investigated family of substances – psoralens – have been known in medicine for many years as amplifiers of DNA sensitivity to UV-A. They can intercalate into DNA structure and form specific covalent bonds with pyrimidines after illumination with 360 nm light (Cole 1971). McGarry et al. (2009) used aminomethyl-4,5',8-trimethylpsoralen (AMT) as a chemical agent that increases sensitivity of *Xenopus* sperm to long wavelength UV light. He showed significantly higher efficiency of enucleation after treatment of sperm with 1μM AMT. Influence of psoralen (PS) on sensitivity of DNA in cells to UV light has long been known and used for skin disease treatment. Cleaver et al. (1985) investigated processes of DNA damage caused by UV-A (360 nm) in presence of PS. He found considerable structural similarity of DNA damage caused by UV-C (254 nm) light and PS treatment followed by UV-A irradiation.

The aim of the present study was to compare different methods of spermatozoa DNA deactivation as an improvement to UV-C treatment which has been the conventional procedure for gynogenetic induction.

MATERIAL AND METHODS

Fish. The experiment was conducted at the Genetic Fisheries Centre, Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic. Spermiation of 15 sterlet males with average weight of 2 kg was induced by injection of carp pituitary extract at 4 mg/kg of body weight 24 h before sperm collection according to Linhart et al. (2000) and Psenicka et al. (2007). Sperm was collected to 20 ml syringes and put on ice until processing.

Sperm with potent spermatozoa motility higher than 70% was chosen for further research. Ovulation of 8 sterlet females (average weight 2.5 kg) was induced by hormonal stimulation with intramuscular injection of carp pituitary suspension in physiological saline solution (0.5 mg per kg body weight) 42 h before the expected ovulation, and 12 h later with the resolving dose (4.5 mg per kg body weight) of identical suspension according to Gela et al. (2008). Ovulated eggs were sampled using the microsurgical incision of oviducts as described by Stech et al. (1999).

Dimethyl sulfate treatment. Dimethyl sulfate (DMS) in concentrations 0.5–15mM was applied to sterlet sperm in order to inactivate DNA. The sperm samples (200 µl) were added to 200 µl of DMS solution with concentrations of 1–30mM. Immediately after addition of DMS, the samples were centrifuged at 200 *g* for 6 min. After centrifugation the supernatant was replaced by non-activating solution that has similar ionic concentrations to native seminal fluid (Psenicka et al. 2008) and the centrifugation was repeated. After the second centrifugation the supernatant was replaced similarly and the samples were stored on ice until fertilization assays.

EB, PS, and AMT treatment. PS, AMT, and EB were applied to increase sensitivity of spermatozoa to longwave UV-A (360 nm). 200 µl of PS solutions in concentrations 1, 2.5, 5, 18, 32, and 200µM, EB in concentrations 1, 5, 10, 20, and 100µM, or AMT in concentrations 0, 1, 5, 10, 25, 50, and 100µM were added to the sperm sample (200 µl) and stored at 4°C. After 2–5 min of incubation the samples were poured on Petri dish with 50 mm diameter that corresponds to approximate sample depth of 0.2 mm. Then the samples were irradiated by UV-A light (360 nm) for 180 s at 5 W/m^2 (900 J/m^2). Intensity of irradiation was measured by Black-Ray UV meter J-221 (Ultra-Violet Products Ltd., Cambridge, UK). UV-C (253 nm) irradiation was conducted by UV crosslinker CL-1000 (Ultra-Violet Products Ltd.), with light intensity 45 W/m^2. Irradiated samples were transferred to Eppendorf tubes covered with foil and kept on ice.

Table 1. Curvilinear velocity of motile spermatozoa after treatment with DMS, AMT, PS or EB 30 s after activation

Chemicals	Concentration	UV-A (360 nm), J/m^2	Average curvilinear velocity ± SD (µm/s)
–	0	0	188.8 ± 9.6
–	0	900	158.1 ± 8.3
PS	10µM	900	144.2 ± 10.1
PS	18µM	900	136.9 ± 11.7
PS	32µM	900	120.4 ± 11.5
PS	50µM	900	0.0
DMS	25mM	0	168.4 ± 8.7
DMS	30mM	0	164.4 ± 10.4
DMS	35mM	0	162.1 ± 6.5
DMS	50mM	0	0.0
EB	20µM	900	140.0 ± 9.0
EB	50µM	900	132.2 ± 6.8
EB	100µM	900	129.6 ± 5.2
EB	200µM	900	0.0
AMT	0µM	900	167.4 ± 34.3
AMT	0.5µM	900	170.6 ± 40.9
AMT	1µM	900	162.3 ± 44.9
AMT	5µM	900	178.0 ± 26.2
AMT	10µM	900	171.5 ± 24.2
AMT	25µM	900	173.3 ± 24.2
AMT	50µM	900	153.4 ± 26.8
AMT	100µM	900	128.6 ± 22.1

DMS = dimethyl sulfate, AMT = 4'-aminomethyl-4,5',8-trimethylpsoralen, PS = psoralen, EB = ethidium bromide, SD = standard deviation

Sperm motility and velocity investigations. Spermatozoa motility was triggered by dilution with non-chlorinated tap water at 1 : 50. Spermatozoa motility and velocity were assessed according to Linhart et al. (2000) using SONY SSC DC50AP video camera (SONY, Tokyo, Japan) coupled to Olympus BX-41 microscope (Olympus Corp., Tokyo, Japan) with stroboscopic light source (50 Hz). Velocity and motility were assessed at 30 s post-activation. The successive positions of the video recorded spermatozoa heads were analyzed from five video frames using Olympus MicroImage software (Version 4.0.1., 1999, for MS Windows with a special macro by Olympus C & S). 20–40 spermatozoa were counted per each frame. Spermatozoa velocity (μm/s) was calculated based on length of spermatozoa traces, calibrated for magnification.

Comet assay. Sperm suspension after treatment was diluted with pre-chilled PBS 1 : 250 and stored at 4°C. Agarose gel (0.8% NuSieve GTG low melting point agarose OxiSelect™; Cell Biolabs, Inc., San Diego, USA) was melted at 90°C and chilled to 37–40°C. Agarose gel was mixed with diluted sperm at the ratio 9 : 1. Microscope slides (OxiSelect™; Cell Biolabs, Inc.) were used for the assay. Immediately after mixing, 50 μl of suspension was pipetted to each well on the slide and spread over the well. Slides were stored at 4°C until solidification of the agarose gel, and then immersed in lysis solution prepared according to the OxiSelect Comet Assay protocol with addition of proteinase K (1 mg/ml of lysis solution). Slides were incubated in lysis solution for 10 h at 37°C. After incubation, lysis solution was carefully aspirated and replaced by pre-chilled electrophoresis solution (90mM Tris Base, 90mM boric acid, 2.5mM EDTA, pH 8.0). After 5 min the slides were carefully transferred to electrophoresis chamber with cold electrophoresis solution. Electrophoresis was carried out at 1 V/cm for 20 min, and then slides were transferred for 5 min to a small container with pre-chilled distilled water. Distilled water was carefully aspirated and slides were air-dried. Once the agarose slide was completely dry, 20 μl per well of Vista Green DNA Staining Solution (OxiSelect™; Cell Biolabs, Inc.) were added. Slides were viewed under Olympus Fluoview microscope (Olympus Corp., Tokyo, Japan) using filters with 450–480 nm excitation wavelengths and recorded by SONY DXC-9100D videocamera (SONY, Tokyo, Japan). The DNA damage in spermatozoa was evaluated using CASP freeware (Version 1.2.3., 2001).

Fertilization and hatching rate. To verify the DNA inactivation for perspective application in gynogenesis induction, solutions with 200 μl of sperm of each sample were added to 20 ml of water from the incubation system and immediately mixed with 5 g of eggs (350–375 eggs). Eggs were activated during 2 min with gentle stirring at 16°C, and then distributed into 3 Petri dishes. After sticking the eggs to the bottom, Petri dishes were immersed into hatching trays in an incubation system (Linhart et al. 2003) and kept at 16°C until hatching. No gynogenetic induction was done, evaluation was based on production of haploid larvae. Hatching rates were estimated as percentage of hatched larvae relative to the amount of eggs used for fertilization.

Ploidy level estimation. Hatched larvae were taken to verify the haploid level. Ploidy was estimated using Partec CCA flow cytometer (Partec GmbH, Münster, Germany) according to Lecommander et al. (1994). Caudal part of each larva was used for determination of relative DNA content per cell. The untreated *Acipenser ruthenus* larvae with ploidy level equal to evolutionary tetraploid (Havelka et al. 2011) were taken as a control group. Separated cells were stained with fluorescent DNA dye, DAPI (4', 6- diamidino-2-phenylindol) which easily passed through the nuclear membrane and bound to base pair sequences in DNA.

Statistical analysis. All values were expressed as mean values ± standard deviation (SD). Data were tested for normal distribution and analyzed by STATISTICA software (Version 9.0, 2009), using One-Way ANOVA, followed by a Tukey's test for comparisons of means. The level of significance was set at $P = 0.05$.

RESULTS AND DISCUSSION

The strong influence of DMS and PS on the motility parameters of spermatozoa was found at concentrations significantly affecting the DNA integrity (Table 1, Figures 1, 3, 4).

Treatment with EB or AMT followed by UV-A irradiation showed slower decrease of spermatozoa motility with increasing concentrations compared to other substances. Namely UV-A irradiation led to significant decrease of motility in the case of AMT, EB, and PS.

Although DMS concentrations of 25–35mM affected spermatozoa motility and velocity less than the treatment with EB or PS followed by UV-A irradiation, presence of 50mM of DMS totally

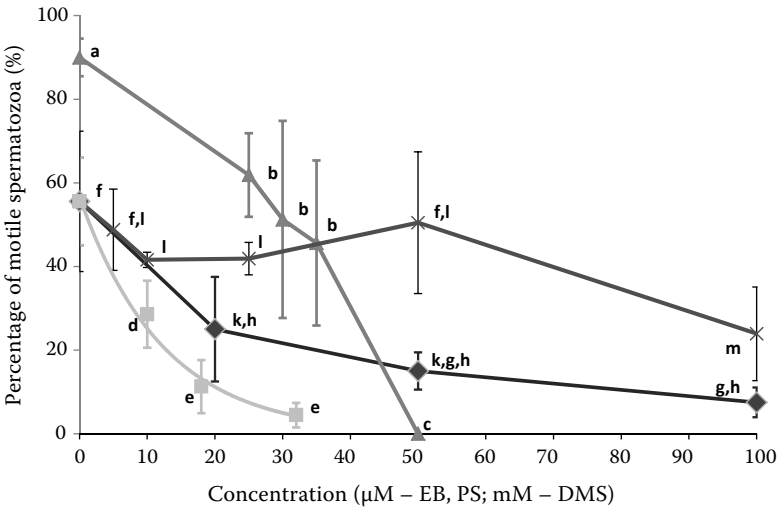

Figure 1. Percentage of motile spermatozoa at 30 s after activation

sperm treated with DMS (▲); PS followed by UV-A (■); AMT followed by UV-A (×); EB followed by UV-A (♦)

DMS = dimethyl sulfate, PS = psoralen, AMT = 4'-aminomethyl-4,5',8-trimethylpsoralen, EB = ethidium bromide

[a–h, k–m]statistically different groups; $P < 0.05$ (ANOVA, Tukey's test)

inhibited spermatozoa motility which makes the application of this treatment limited.

As a next step in the assessment of the perspectives of application of AMT, DMS, EB, PS, and UV irradiation for DNA inactivation, the influence of these factors on DNA integrity was evaluated (Figures 2, 3). The highest level of DNA fragmentation was achieved by treatment with DMS at concentration of 100mM, but as seen in Figure 1, motility of spermatozoa was totally inhibited already at 50mM of DMS. Nevertheless, treatment with DMS at concentrations 25–35mM did not completely inhibit motility and significantly increased the level of DNA damage compared to control. Longwave UV-A irradiation (360 nm, 900 J/m^2) did not cause significant damage to the DNA of sperm cells (Figure 3B, C) in contrast to UV-C (260 nm) irradiation, where the same irradiation dose caused 5 times higher DNA damage compared to control (Figure 3D). This correlates with the dependence of the level of pyrimidine dimer formation on wavelenghts of UV-light discribed by Enninga et al. (1986).

Additionally it was shown that damage caused by AMT + UV-A, PS + UV-A, and EB + UV-A treatments did not originate only from UV-A irradiation or the substances, but actually it was caused by the combination of each substance with UV-A irradiation. However, despite the lower sensitivity of DNA to UV-A light, motility apparatus retained highly sensitive to UV-A. Therefore, although the addition of AMT, EB, and PS caused increase of the DNA sensitivity to the longwave UV-A light (Figures 2, 3B, C), it also negatively influenced sperm motility (Figure 1), which did not allow to obtain reasonable fertilization leading to higher survival rates (Figure 4A, B). On the other hand, lower influence of AMT treatment on motility systems and higher DNA damage resulted in higher percentage of gynogenotes in progeny (Figure 5).

Flow cytometric analysis of larvae hatched from the eggs activated by DMS treated spermatozoa did not show any haploid larvae up to the dose of 5mM DMS; the observation is consistent with the result of gynogenesis induction in peled and rain-

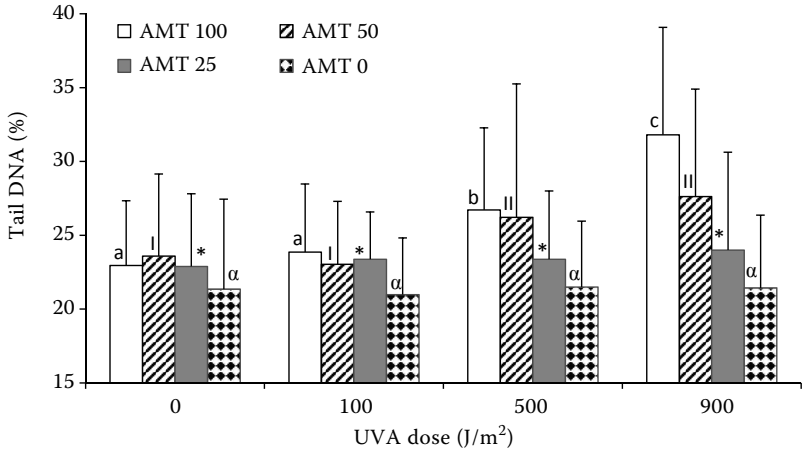

Figure 2. Level of DNA damage in spermatozoa measured by comet assay after treatment with 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) (0, 25, 50, 100μM) followed by UV-A (0, 100, 500, 900 J/m^2)

[a–c; I–II; *; α]statistically different groups; $P < 0.05$ (ANOVA, Tukey's test)

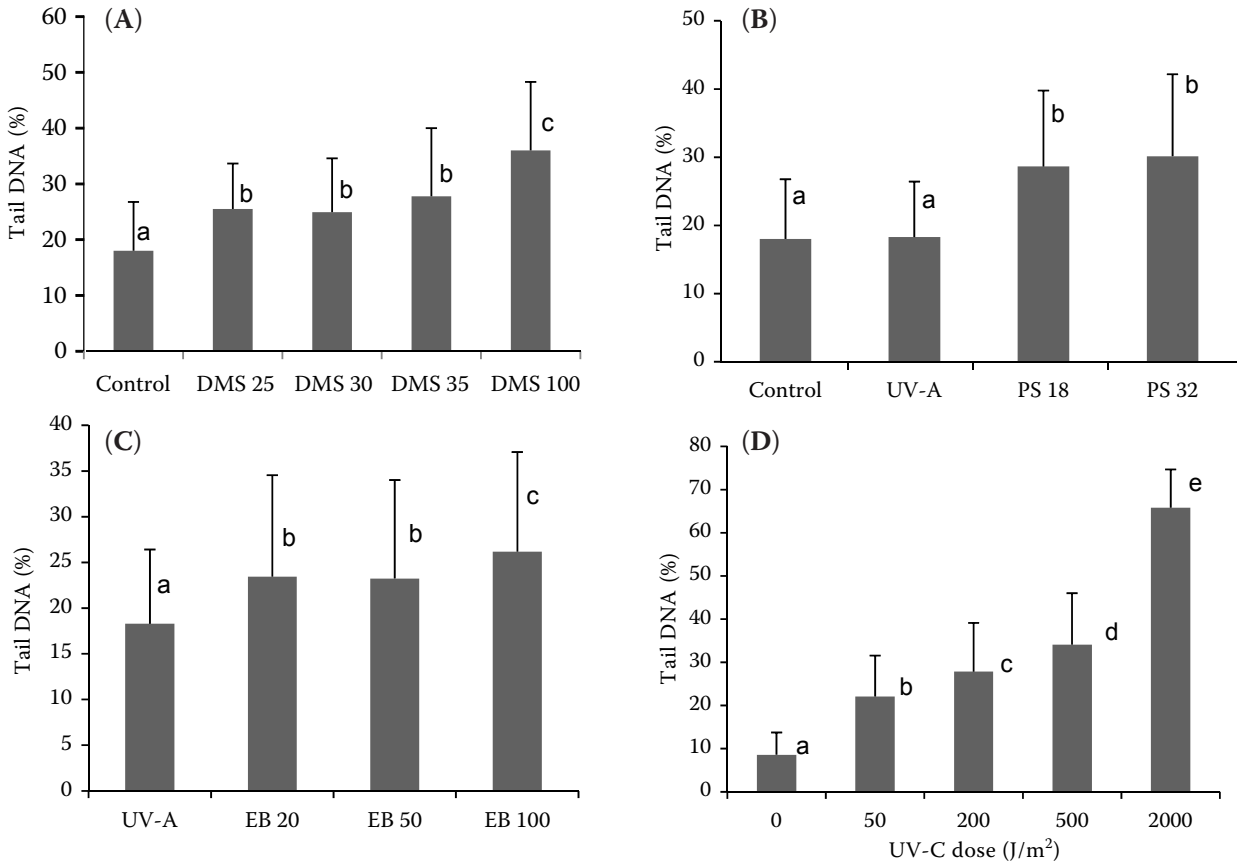

Figure 3. Level of DNA damage in spermatozoa measured by comet assay after treatment with DMS (25, 30, 35, 100mM) (**A**); PS (18, 32μM) + UV-A (900 J/m^2) and UV control (UV-A irradiation 900 J/m^2) (**B**); EB (20, 50, 100μM) + UV-A (900 J/m^2) (**C**); UV-C (254 nm) (**D**)

DMS = dimethyl sulfate, PS = psoralen, EB = ethidium bromide

[a–e]statistically different groups; $P < 0.05$ (ANOVA, Tukey's test)

bow trout by Tsoi (1969). We suppose that such a low survival rate in the experiment of Tsoi could be explained by the lack of the method on separating spermatozoa from DMS solution after treatment and preventing the direct effect of DMS on eggs.

Purely haploid progeny was obtained after treatment with doses of about 30mM of DMS (Figure 4B) but only few spermatozoa were motile at this concentration. This was the reason to assume that DMS did not damage DNA sufficiently for prospective induction of gynogenesis at concentrations safe for sperm motility apparatus.

Similarly to DMS, ploidy investigations of larvae developed from sperm treated with PS at doses up to 18μM and EB up to 10μM and followed by UV-A irradiation at the dose of 900 J/m^2 did not reveal any significant amount of haploid larvae (Figure 4B). That is consistent with our result of comet assay and sperm motility investigation. In general, the amount of hatched larvae correlated with sperm motility analysis (Figure 4A) and showed strong negative correlation between the doses of chemical agents and hatching rate.

The highest percentage of putative haploid gynogenetic larvae (up to 19.81%) was obtained after treatment of sperm with AMT at the dose of 100μM followed by UV-A irradiation at 900 J/m^2. Further optimizations of AMT treatment led us to possible substitution of UV-irradiation method by the chemical treatment. However, chemical treatment of spermatozoa may impact on the progeny development, therefore further investigation is required.

CONCLUSION

All tested chemicals can be used for induction of gynogenesis in sterlet. Nevertheless DMS showed low efficiency of DNA inactivation and selectiveness of the influence on sterlet sperm. The DMS concentrations completely inactivating sperm DNA

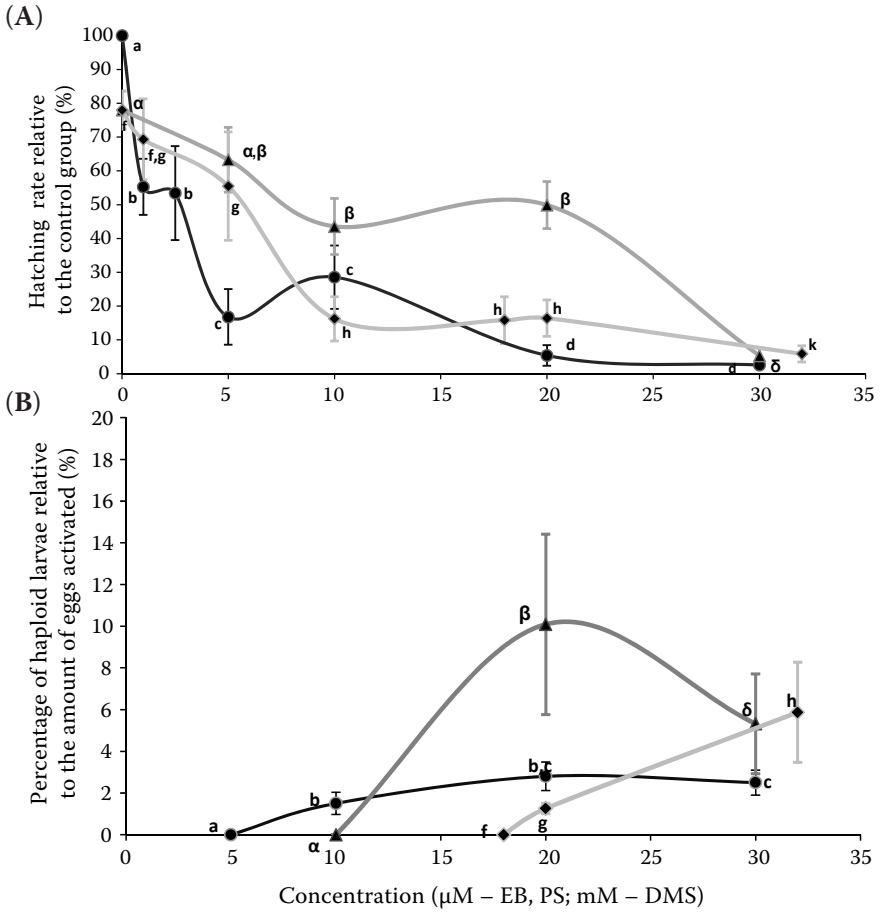

Figure 4. Hatching rate (relative to the survival of control group) (**A**) and percentage of putative gynogenetic larvae (relative to the amount of eggs) (**B**) after treatment of sperm with DMS (●); PS + UV-A (900 J/m^2) (♦); EB + UV-A (900 J/m^2) (▲)

DMS = dimethyl sulfate, PS = psoralen, EB = ethidium bromide

superscript letters indicate significant differences between means (ANOVA, $P < 0.05$)

greatly reduced the number of motile spermatozoa thereby creating a limit to the DMS effectiveness at about 2–3% of the control hatching rate. Treatment with PS and EB also showed a strong overwhelming impact on the sperm motility. At the same time, compared with a widely used UV-C irradiation treatment, it did not prove to be more efficient. On the other hand, treatment with AMT showed quite low influence on sperm motility and as a result could be used as substitution for UV-C

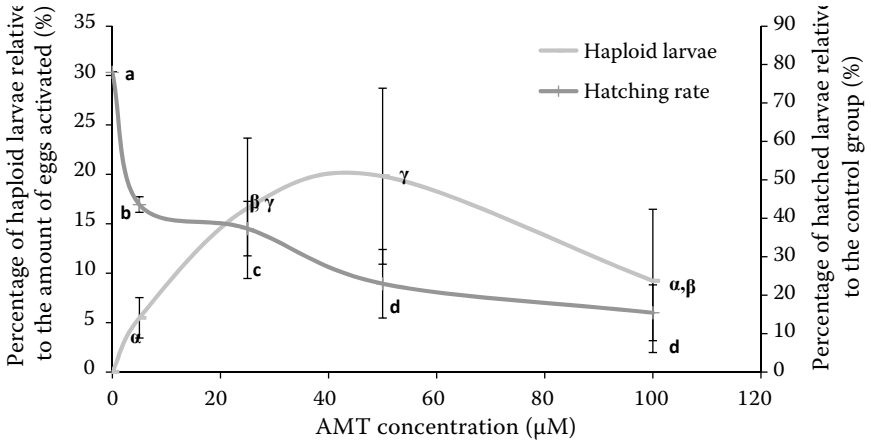

Figure 5. Hatching rate and percentage of haploid larvae in progeny obtained from sperm treated with 4'-aminomethyl-4,5'8-trimethylpsoralen (AMT) in concentrations of 0, 5, 25, 50, 100μM followed by UV-A irradiation (900 J/m^2)

$^{a–d, \alpha–\gamma}$statistically different groups; $P < 0.05$ (ANOVA, Tukey's test)

irradiation. Despite the obscure results of chemical induction of gynogenesis, the finding of new ways of DNA inactivation could help us substitute the difficultly optimizable UV-irradiation method by the chemical treatment.

REFERENCES

Billard R., Lecointre G. (2001): Biology and conservation of sturgeon and paddlefish. Reviews in Fish Biology and Fisheries, 10, 355–392.

Cariello N.F., Keohavong P., Sanderson B.J., Thilly W.G. (1988): DNA damage produced by ethidium bromide staining and exposure to ultraviolet light. Nucleic Acids Research, 16, 4157.

Christensen J.M., Tiersch T.R. (1994): Standardization of ultraviolet irradiation of channel catfish sperm. Journal of the World Aquaculture Society, 25, 571–575.

Cleaver J.E., Killpack S., Gruenert D.C. (1985): Formation and repair of psoralen-DNA adducts and pyrimidine dimers in human DNA and chromatin. Environmental Health Perspectives, 62, 127–134.

Cole R.S. (1971): Psoralen monoadducts and interstand cross-links in DNA. Biochimica et Biophysica Acta, 254, 30–39.

Dietrich G.J., Szpyrka A., Wojtczak M., Dobosz S., Goryczko K., Zakowski L., Ciereszko A. (2005): Effects of UV irradiation and hydrogen peroxide on DNA fragmentation, motility and fertilizing ability of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Theriogenology, 64, 1809–1822.

Enninga I.C., Groenendijk R.T.L., Filon A.R., van Zeeland A.A., Simons J.W. (1986): The wavelength dependence of UV-induced pyrimidine dimer formation, cell killing and mutation induction in human dipolid skin fibroblasts. Carcinogenesis, 7, 1829–1836.

Flynn S.R., Matsuoka M., Reith M., Martin-Robichaud D.J., Benfey T.J. (2006): Gynogenesis and sex determination in shortnose sturgeon, *Acipenser brevirostrum* Lesuere. Aquaculture, 253, 721–727.

Fontana F., Tagliavini J., Congiu L. (2001): Sturgeon genetics and cytogenetics: recent advancements and perspectives. Genetica, 111, 359–373.

Fopp-Bayat D. (2010): Meiotic gynogenesis revealed not homogametic female sex determination system in Siberian sturgeon (*Acipenser baeri* Brandt). Aquaculture, 305, 174–177.

Fopp-Bayat D., Jankun M., Woznicki P., Kolman R. (2007): Viability of diploid and triploid larvae of Siberian sturgeon and bester hybrids. Aquaculture Research, 38, 1301–1304.

Gela D., Rodina M., Linhart O. (2008): Controlled reproduction of sturgeon. Edition methodology. Research Institute of Fish Culture and Hydrobiology, University of South Bohemia in České Budějovice, Vodňany, 78, 1–24. (in Czech).

Havelka M., Kaspar V., Hulak M., Flajshans M. (2011): Sturgeon genetics and cytogenetics: a review related to ploidy levels and interspecific hybridization. Folia Zoologica, 60, 93–103.

Keyvanshokooh S., Gharaei A. (2010): A review of sex determination and searches for sex-specific markers in sturgeon. Aquaculture Research, 41, 1–7.

Lecommander D., Haffray P., Philippe L. (1994): Rapid flow cytometry method for ploidy determination in salmonid eggs. Aquaculture and Fisheries Management, 25, 345–350.

Linhart O., Mims S.D., Gomelsky B., Hiott A.E., Shelton W.L., Cosson J., Rodina M., Gela D. (2000): Spermiation of paddlefish (*Polyodon spathula,* Acipenseriformes) stimulated with injection of LHRH analogue and carp pituitary powder. Aquatic Living Resources, 13, 455–460.

Linhart O., Rodina M., Gela D., Kocour M., Rodriguez M. (2003): Improvement of common carp artificial reproduction using enzyme for elimination of eggs stickiness. Aquatic Living Resources, 16, 450–456.

Matselyukh B.P., Matselyukh D.Y., Kovalska V.B., Volkova K.D., Kryvorotenko D.V., Yarmoluk S.M. (2005): Studies of mutagenic activity of fluorescent DNA sensitive monomethinecyanine and carbocyanine dyes in Ames test. Ukrainica Bioorganica Acta, 2, 27–34.

McGarry T.J., Bonaguidi M., Lyass L., Kessler J.A., Bodily J.M., Doglio L. (2009): Enucleation of feeder cells and egg cells with psoralens. Developmental Dynamics, 238, 2614–2621.

Mims S.D., Shelton W.L. (1998): Induced meiotic gynogenesis in shovelnose sturgeon. Aquaculture International, 6, 323–329.

Omoto N., Maebayashi M., Adachi S., Arai K., Yamauchi K. (2005): Sex ratios of triploids and gynogenetic diploids induced in the hybrid sturgeon, the bester (*Huso huso* female × *Acipenser ruthenus* male). Aquaculture, 245, 39–47.

Psenicka M., Alavi S.M., Rodina M., Gela D., Nebesarova J., Linhart O. (2007): Morphology and ultrastructure of Siberian sturgeon (*Acipenser baerii*) spermatozoa using scanning and transmission electron microscopy. Biology of the Cell, 99, 103–115.

Psenicka M., Hadi Alavi S.M., Rodina M., Cicova Z., Gela D., Cosson J., Nebesarova J., Linhart O. (2008): Morphology, chemical contents and physiology of chondrostean fish sperm: a comparative study between Siberian sturgeon (*Acipenser baerii*) and sterlet (*Acipenser ruthenus*). Journal of Applied Ichthyology, 24, 371–377.

Recoubratsky A.V., Grunina A.S., Barmintsev V.A., Golovanova T.S., Chudinov O.S., Abramova A.B., Panchenko N.S., Kupchenko S.A. (2003): Meiotic gynogenesis in the stellate and Russian sturgeons and sterlet. Russian Journal of Developmental Biology, 34, 92–101.

Shelton W.L., Mims S.D. (2012): Evidence for female heterogametic sex determination in paddlefish *Polyodon spathula* based on gynogenesis. Aquaculture, 356–357, 116–118.

Stech L., Linhart O., Shelton W.L., Mims S.D. (1999): Minimally invasive surgical removal of ovulated eggs from paddlefish (*Polyodon spathula*). Aquaculture International, 7, 129–133.

Tsoi R.M. (1969): Effect of nitrosomethylurea and dimethylsulfate on the sperm of the rainbow trout (*Salmo irideus*) and the white fish (*Coregonus peled*). Doklady Akademii Nauk SSSR, 189, 411.

Tsoi R.M. (1974): Chemical gynogenesis in *Salmo irideus* and *Coregonus peled*. Soviet Genetics, 8, 275.

Uwa H. (1965): Gynogenetic haploid embryos of the medaka (*Oryzias latipes*). Embryologia, 9, 40–48.

Van Eenennaam A.L., Van Eenennaam J.P., Medrano J.F., Doroshov S.I. (1999): Evidence of female heterogametic genetic sex determination in White sturgeon. Journal of Heredity, 90, 231–233.

Waring M.J. (1965): Complex formation between ethidium bromide and nucleic acids. Journal of Molecular Biology, 13, 269–282.

Received: 2013–09–03
Accepted after corrections: 2014–02–19

Corresponding Author

MSc. Ievgen Lebeda, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic

Phone: +420 387 774 753, e-mail: ilebeda@frov.jcu.cz