

***In Vitro* Storage of Ova of Persian Sturgeon, *Acipenser persicus* in Ovarian Fluid: the Changes in the pH and Osmolality of the Ovarian Fluid, Fertilization and Hatching Rates**

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Abstract: The unfertilized ova of Persian sturgeon, *Acipenser persicus*, were stored in the ovarian fluid at 18°C and fertilized after 0 (control), 3, 6, 9 and 12 hours post stripping using fresh and pooled sperm obtained from three males. In addition, changes in the osmolality and pH of ovarian fluid during storage were recorded. After 3 h of storage, no significant differences were found between fertilization and hatching rates in comparison with control. But, after 6h, 9h and 12 h of storage, fertilization and hatching rates significantly decreased in comparison with control. After 3 h and 6 h of storage, no significant differences were found between values of osmolality of ovarian fluid compared to control. But, after 9 h and 12 h of storage values of osmolality increased significantly compared to control. Also, significant negative correlations were found between osmolality of ovarian fluid and fertilization and hatching rates. The value of pH of ovarian fluid significantly decreased after 6 h of storage compared to control. This decline in the values of pH continued towards the end of the experiment. Also, significant positive correlations were found between pH of ovarian fluid and fertilization and hatching rates. Results showed that the ovulated eggs of Persian sturgeon can be held in ovarian fluid at 18 °C up to 3 hours without significant loss of hatchability and fertilization rate. As well as, the decline of pH and increase of osmolality of ovarian fluid may be two limiting factors for short term storage of ova of Persian sturgeon.

Key words: short-term storage, ova, ovarian fluid, *Acipenser persicus*, osmolality, pH

INTRODUCTION

Attempts to freeze fish ova have been unsuccessful, because of inadequate dehydration and toxicity of cryoprotectants (Rana, 1995). As a consequence, short-term storage protocols using positive temperatures in ovarian fluid and artificial media have been developed. Short-term preservation aimed at increasing post spawning gamete longevity, may improve hatchery management, minimize problems resulting from inbreeding and provide synchronous brooder maturation (Bromage and Roberts, 1995).

The Persian sturgeon, *Acipenser persicus*, is a vulnerable anadromous fish considered to biological conservation program in southern part of Caspian Sea (Kiabi *et al.*, 1999). It is valuable to measure storage capacity of unfertilized eggs of this commercially and ecologically valuable species. The objectives of present study were to: (a) evaluate the viability of unfertilized ova of Persian sturgeon during *in vitro* storage in ovarian fluid (b) investigate the changes of pH and osmolality of ovarian fluid as two physiochemical properties during storage period.

MATERIAL AND METHODS

The experiment was carried out at Shahid Beheshti Artificial Sturgeon Propagation and Rearing Center, Iran, Rasht. Three females (TL= 168.3 ± 7.6 cm, Weight = 18.2 ± 0.9 Kg) and males Persian sturgeon (TL= 112.4 ± 3.6 cm, Weight = 13.8 ± 0.4 Kg) were captured during spawning season (March to May). Just before experiment, to induce the ovulation and spermiation, the females and males were injected intramuscularly with

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acetone-dried sturgeon pituitary homogenate according to water temperature (2 mg.kg⁻¹ body weight and 1 mg.kg⁻¹ body weight in one dose for females and males, respectively). In this regard, after 16 hours females were ovulated and the eggs were collected by cutting of abdomen from the anus towards the pectoral fins. Then, eggs were separated from ovarian fluid by a net with tiny meshes.

To store eggs in ovarian fluid, 25 g of eggs (5g = approximately 260 eggs) were gently weighted and equally distributed in 5 circular plastic plates (5g in each circular plastic plate). Subsequently, 3 mL of ovarian fluid was added to each plate. In this experiment, eggs and ovarian fluid of each female was stored separately. The plates were held in an Incubator (Model 35823-260 – Each) with a constant temperature (18 °C), mimic to water temperature of broodstocks pond until fertilization. The egg batches were fertilized on times 0 (control), 3, 6, 9 and 12 h after stripping. On each time, sperm from each male was collected by hand stripping and its quality assessed by means of density observations and motility test (Dettlaff *et al.*, 1993). Motility was estimated under light microscope (×400) as a percentage of motile spermatozoa during first 30s after dilution in water (1:40). In order to reduce the variability of sperm fertilization potency due to their different origin, the semen from 3 males were pooled. Before fertilization, the ovarian fluids from egg batches were removed and their pH and osmolality were measured with an Osmometer (Melting Point Osmometer Nr 961003, Roebling Company, Berlin, Germany) and a semi-microelectrode (SM102 pH Meter), respectively. After eliminating adhesiveness of egg membranes, fertilized eggs were placed in Yoshchenko incubators with running water (28 L.min⁻¹, 17-18 °C). To eliminate adhesiveness of egg membranes, 3 minutes after mixing of ova and semen, a volume of 600 ml clay suspension (10%) was added to this mixture. Afterward, the eggs were mixed slowly by hand for 15 minutes and then eggs were washed by freshwater. This process was repeated 3 times and therefore, the elimination of adhesiveness lasted 45 minutes.

Fertilization rate was determined according Dettlaff *et al.* (1993). For this purpose, 70-90 eggs were sampled randomly from each incubator in the time of second mitosis division which usually the second division occurs 3-4 hour after fertilization in 17-18 °C in sturgeons. Afterward, sampl each egg batch were fixed in formalin solution (5 mL formalin (40%) + 45 mL water) and then, eggs were investigated under a Stereomicroscope (Meiji EMZ-1). The eggs with four blastomeres in animal pole were considered as fertilized eggs and others without cleavage, with more than four blastomeres and damaged eggs were considered as unfertilized eggs. After 6 days of incubation, larvae hatched. Hatching rate was determined by counting the number of free larvae in the trough and the number of eggs that did not hatch after 24 hours (Gisbert and Williot, 2002).

The SPSS software was used for data analysis. The values of pH and osmolality were normal according to Kolmogorov Smirnov test but because of percentage data (fertilization and hatching rate) did not have a normal distribution, proportional data were converted by angular transformation ($\arcsin\sqrt{p}$). Afterward, data were analyzed by ANOVA test and the means were compared by using the Tukey test. The data were expressed as mean ± sd. Also, the relationships between pH and osmolality with fertilization and hatching rates were tested using the bivariate correlation coefficients of Pearson. Then, Linear and non-linear regression models were investigated using regression fits.

RESULTS AND DISCUSSION

After 3 h of storage, no significant differences were found between fertilization and hatching rates in comparison with control ($p>0.05$). But, after 6, 9 and 12 h of storage, fertilization and hatching rates significantly decreased in comparison with control (Figure1, $p<0.05$). After 3 and 6 h of storage, no significant differences were found between values of osmolality of ovarian fluid compared to control ($p>0.05$). But, after 9 and 12 h of storage values of osmolality increased significantly compared to control (Figure2, $p<0.05$). Also, significant negative correlations were found between osmolality of ovarian fluid and fertilization and hatching (Figure3, $p<0.0001$) rates. The value of pH of ovarian fluid significantly decreased after 6 h of storage compared to control ($p<0.05$).

The decline in the values of pH continued until end of the experiment (Figure4). Also, significant positive correlations were found between pH of ovarian fluid and fertilization and hatching (Figure5, $p<0.0001$) rates. The results of present study showed that ovulated eggs of Persian sturgeon can be held in ovarian fluid at 18 °C up to 3 hours without significant loss of hatchability and fertilization rate. But, the fertilization rate and hatchability of stored ova for 6, 9 and 12 h decreased significantly towards the end of experiment.

Gisbert and Williot (2002) observed that ovulated eggs of Siberian sturgeon, *Acipenser baeri*, and Sterlet, *A. ruthenus*, stored in ovarian fluid at 15 °C retained their viability up to 2-4 h, respectively. After such a period of time, eggs become over-ripe and their fertilizability and hatchability were dramatically reduced.

Kjorsvik *et al.* (1990) reported that after stripping, fish eggs gradually undergo changes similar to over-ripening. Therefore, oocyte over-ripening may be evaluated on the basis of the decline in fertilization and hatching rates probably by adverse effects on function of micropyles or factors involving in embryonic development.

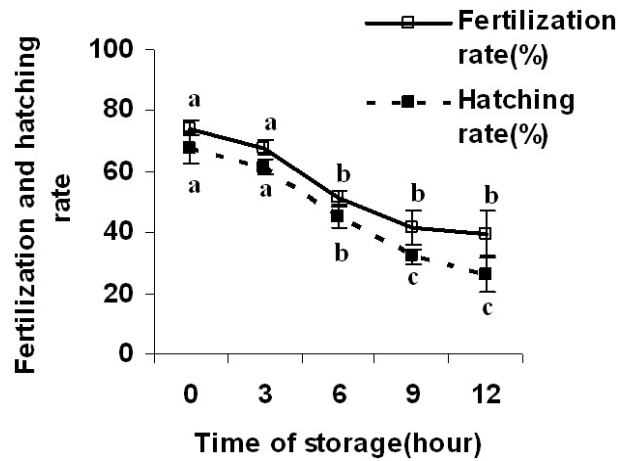


Fig. 1: Changes in the fertilization and hatching rates of Persian sturgeon ova during *in vitro* storage in ovarian fluid (0h= control).

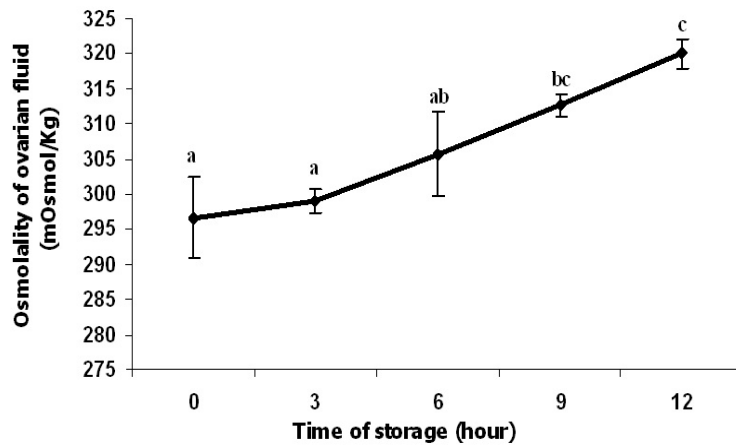


Fig. 2: Changes in the osmolality of ovarian fluid during *in vitro* storage of Persian sturgeon ova (0h= control).

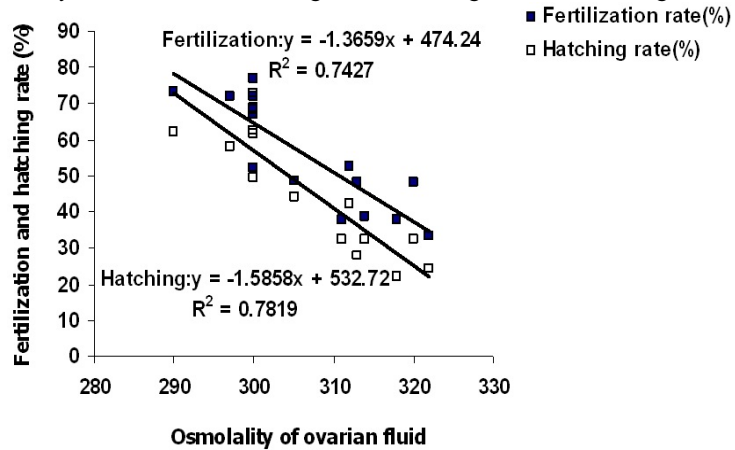


Fig. 3: Relationships between the osmolality of ovarian fluid and the fertilization and hatching rates during *in vitro* storage of Persian sturgeon ova.

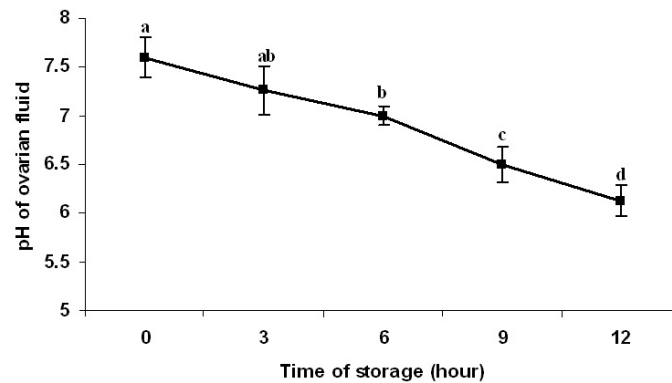


Fig. 4: Changes in the pH of ovarian fluid during *in vitro* storage of Persian sturgeon ova (0h= control).

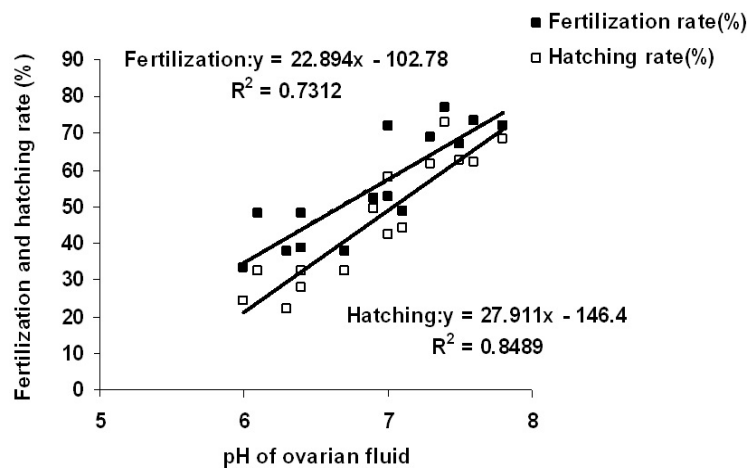


Fig. 5: Relationships between the pH of ovarian fluid and the fertilization and hatching rates during *in vitro* storage of Persian sturgeon ova.

According to the observations of present study, the pH of ovarian fluid decreased with time and it had a close relationship with reduction of eggs quality. In carp, a disturbance in the aerobic respiration process occurs that leads to the production of lactic acid, which accumulates in the ovarian fluid and reduces its pH, with ultimate loss of oocyte membrane integrity (Linhart *et al.*, 1995). The drop in pH reflects an accumulation of CO₂ which binds with water to form carbonic acid (Komrakova and Holtz, 2009). Lahnsteiner (2000) surmised that the decrease in pH observed with overripe eggs might result from an accumulation of compounds derived from eggs such as aspartate aminotransferase, protein and esterified and non-esterified fatty acids during lysis of the eggs rather than leakage from structurally intact eggs.

The increase in the levels of osmolality of ovarian fluid in the present experiment can be attributed to the possible evaporation and loss of cellular material as a result of membrane damage caused by storage conditions during storage. Thus, these agents may increase the osmolality of ovarian fluid out of the tolerable range of eggs and thereby reduce the quality of eggs with time. In Conclusion, The results of present study showed that ovulated eggs of Persian sturgeon can be held in ovarian fluid at 18 °C up to 3 hours without significant loss of hatchability and fertilization rate. Also, the pH and osmolality may be limiting factors for short term storage of ova of Persian sturgeon. Further studies are needed to develop methods to stabilize these factors and investigate of their exact role on egg viability.

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