Turkish Journal of Fisheries and Aquatic Sciences 8: 171-175 (2008)

Effect of Ascorbic Acid Supplementation on Sperm Quality of Rainbow Trout (*Onchorynchus mykiss*)

Mehmet Ali Canyurt[1], Süleyman Akhan[2,*]
[1] Ege University, Fisheries Faculty, Department of Aquaculture, 35100, Bornova, İzmir, Turkey.
[2] Rize University, Fisheries Faculty, Department of Aquaculture, 53100, Rize, Turkey.

* Corresponding Author: Tel.: +90.464 223 33 85; Fax: +90.464 223 41 18;
E-mail: akhan@rize.edu.tr

Received 23 October 2007
Accepted 25 March 2008

Abstract

The aim of the work was to determine the effects of two different doses of ascorbic acid (300 mg kg^{-1} and 800 mg kg^{-1}). They were evaluated on sperm quality of rainbow trout. The results of study showed that 800 mg kg^{-1} ascorbic acid supplemented diet was affected sperm quality positively such as sperm concentration, spermatocrit, sperm motility and fertilizing capacity of sperm. However, feeding with the diet supplemented with 300 mg kg^{-1} ascorbic acid increased sperm motility, but did not significantly affect according to control group.

Key words: ascorbic acid, sperm concentration, sperm motility, fertilization ability.

Introduction

There are several agents affecting gamete quality in fish such as environmental conditions and genetic factors. Nutrition and content of feed (fatty acids, amino acids, minerals and vitamins) are environmental cues that influence reproduction in fish (Bromage, 1995; Izquierdo *et al*., 2001). Vitamin C (ascorbic acid, AA) is also one of the micro nutrient elements in feeding teleost fish. AA plays a critical role in all living organisms. Trout, salmon and other fish species have dietary requirements for AA (Benitez and Halver, 1982).

Although nutritional requirements of fish were examined well for breeding purposes, requirements of essential nutrient elements such as ascorbic acid were not studied in male brood fish either. There are many studies about ascorbic acid requirement of salmonid fish. AA requirements of some salmonid species were determined (Halver *et al*., 1969; Hilton *et al.,* 1978; Sato *et al.,* 1978; Johnston *et al.,* 1989; Sandnes *et al.,* 1992; Waagbø and Sandnes, 1996) for growing. AA requirements of fish were showed variation in respect of fish physiology and their development stage (e.g. larval stage or gonad development) (Dabrowski and Ciereszko, 2001).

AA also plays as a critical role on gamete quality of fish like other vertebrates (Ciereszko and Dabrowski, 2000). Long term feeding, using diets without AA resulted in decreases of sperm concentration, motility and fertilizing ability, and an increase in sperm lipid peroxidation value (Ciereszko and Dabrowski, 1995; Ciereszko *et al*., 1996; Dabrowski and Ciereszko, 1996; Liu *et al*., 1997)

AA concentration was found higher in rainbow trout seminal plasma (30-60 ppm) than blood plasma (1-10 ppm). This suggests that AA may be more important as an antioxidant in male gonad (Ciereszko and Dabrowski, 1995). Numerous studies were made on human infertility; positive effect of AA on sperm quality of men was demonstrated (Dawson *et al*,. 1992). Antioxidants such as AA protect germ cells against to DNA damage, and oxidation seminal plasma proteins with reactive oxygen radicals (Fraga *et al*., 1991; Liu *et al*., 1995).

Teleost fish cannot synthesise AA like other vertebrate because of lack of active gulonolactone oxidase which is terminal enzyme in AA synthesis pathway (Ciereszko *et al*., 1999). Therefore, AA requirements of fish should be provided with diet. On the other hand, AA is very labile and thus readily destroyed in the manufacturing process, especially in extruded feeds. Therefore, it is not usually added to multivitamin premixes for fish feeds. Various coated forms of ascorbic acid, such as ethylcellulose or fat-coated products, have been used to increase retention of the vitamin in fish feeds. Nevertheless, approximately 50 percent of the supplemental AA is destroyed during the manufacture of feeds (Lovell and Lim, 1978). So, additional AA should be supplemented to commercial feed to ensure an adequate concentration of the vitamin.

The aim of this study was to evaluate the effects of AA supplemented diet at 300 mg kg^{-1} and 800 mg kg^{-1} feed up to spawning period (three months; from August to November 2003) on sperm quality of 2^{+}-3^{+} years old male rainbow trout such as motility, motility duration, spermatozoa concentration, spermatocrit and fertilizing capacity.

Material and Methods

Experimental Fish and Fish Meal

120 adult male rainbow trout (*Oncorhynchus mykiss*) at the age of , 2^{+}- 3^{+}, altering 1,200 and 1,800 g initial weight were used in the study. Trials were performed in a private hatchery located in İzmir, Turkey. Fish were divided into six groups in duplicates (AA300, AA800 and AAC: control). They were stocked in rectangular concrete ponds (5x2x1 m dimension) in summer (June 2003) and were fed *ad libitum* with a 6 mm commercial feed (Table 1) supplemented with 300 mg kg^{-1} and 800 mg kg^{-1} AA and no AA added into control diet (6 mm commercial pellets) twice a day until spawning season (November 2003).

Adding Ascorbic Acid

The feed was weighed and AA was added at dose of 300 mg kg^{-1} and 800 mg kg^{-1} dry feed and mixed properly, and then water was sprayed to those mixtures in order to absorb water soluble AA. This procedure was repeated before each meal and the feed was presented to experimental fish immediately. Approximate biochemical components of AA supplemented and non AA supplemented feeds were analyzed according to Association of Official Analytical Chemists (AOAC, 1990).

Sperm Collection and Measurement of Sperm Quality

Total of 10 male fish were selected randomly from each group and all milt were stripped in a dry and cool glass vials. Before stripping, anal region of fish were cleaned by dry towel. Anal and seminal duct excreted for avoiding urea and faces contamination. All sperm manipulations were performed on ice. Semen sampled for quality estimate was used within 6-8 h after stripping.

Spermatozoa concentration was estimated as fallows: 25 µl milt was diluted with artificial seminal plasma (ASP) (1.6 mM $CaCl_2$, 120 mM NaCl, 30 mM KCl, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, pH 8) and counted using Thoma counting chamber at 400x magnification as described by Konuk (1975). Sperm concentration measurements were done twice for each sample.

Micro haematocrit tubes (75 mm length, 1.1-1.2 internal diameter) were used for determination of spermatocrit. The duplicated tubes for each sample were filled up with milt and one side of tubes were covered with glass putty and plaster. They were centrifuged at 2600 RPM for 30 min and measured using haematocrit ruler.

Sperm was diluted using ASP at ratio of 1:100. 10 µl diluted semen was put in bovine serum albumin (BSA) coated microscope slide. The percent motility of each sperm sample was estimated using light microscopy at 400x magnification immediately after addition of 20 µl of farm water used as an activating solution. During spermatozoa activation, immotile sperm cells (ISC) were counted, and when the activation gets stopped, whole sperm cells (WSC) were counted in per microscopic area by naked eyes, and then motile sperm cells (MC) were calculated as MC=WSC-ISC. Motility was determined as the percentage of sperm actively moving forward according to that formula: motility % (M) = MC / WSC x 100. To determine duration of motility, 10 µl diluted semen was activated under 100 x magnification. The time interval that is beginning from 5 h after activation of sperms until when all sperms get stopped, was recorded as motility duration. Sperm motility and motility duration measurements were done twice for each sample.

In order to determine fertilizing capacity of sperm, the eggs were stripped into large plastic bowl and mixed from three female broods fed with no vitamin supplemented diet. Stripped eggs were obtained from the female using standard stripping procedure. Sampled eggs were initially placed in a large bowl, and the ovarian fluid was allowed to drip away. The eggs were weighed and divided into aliquots of 500 and placed in polyethylene cups. Semen (1 ml from each male) were added to the cups and gently swirled for 1 min and then allowed to stand for 5 min to allow fertilization. After this process, clean rearing water was added into eggs and left for 30 min. Then fertilized eggs were transferred to incubation trays. The incubation tray was kept in total darkness during the initial 14- d post-fertilization period. As an indicator of reproductive success, embryological development was assessed on day 16

Table 1. Composition of experimental diets

Dry matter basis	Diets		
	AA300	AA800	AAC
Crude Protein (%)	45.1	45.1	45.1
Crude Fat (%)	15.3	15.3	15.3
Ash	12.0	12.0	12.0
Humidity (%)	10.0	10.0	10.0
Energy (kcal/kg)	3800	3800	3800
AA supplementation (mg kg^{-1})	300	800	No AA added

by measuring the percentage of eyed eggs in each incubation tray. All fertilization trials were done in twice for each sperm sample and accepted mean of those samples results.

Statistical Analysis

Statistical analyses were carried out using SPSS Version 10.0 software. Analysis of variance and a least significant difference (LDS) multiple comparisons test were done for determination of significant differences between groups. Significant differences between groups were tested at the 95% confidence interval. Unless otherwise indicated, mean values ± S.E are presented.

Results

Sperm quality parameters obtained from experimental groups and control group were given in Table 2. Sperm concentration ranged from $8.87x10^9$ cell ml^{-1} to $15.3\ x10^9$ cell ml^{-1}. Low sperm concentration was detected in control; mean sperm number was counted as $9.875x10^9 \pm 0.23x10^6$. There were significant differences in mean concentrations between AA supplemented groups (AA300 and AA 800) and control group (AAC) ($P<0.05$); but there were no significant differences detected between groups fed with AA supplemented diets; group AA300 had a mean concentration of $11.88\ x10^9 \pm 0.335\ x10^6$ cell ml^{-1} and group AA800 males $11.75\ x10^9 \pm 0.35\ x10^6$ cell ml^{-1}. Spermatocrit values also increased with AA supplementation. Average spermatocrit values of the groups AA300 (23.6±0.95%) and AA800 (25.80±0.99%) were found significantly higher than those of control (20.1±1.25%). Motility duration was recorded as 38.4±1.58 s in group AA300, 36.9±0.85 s in group AA800 and 35.4±1.6 s in group AAC. There were no significant differences between the groups. The highest motility rate was recorded in group AA800 as 91.42% and the lowest motility was detected in control group as 70.02%. Mean sperm motilities in groups AA300, AA800 and AAC were 81.19±2.45, 88.99±1.07 and 78.25±3.63, respectively. At the eyed stage, mean survival rate of ova was found as 83.5±2.16% in group AA300, 87.8±2.05% in group AA800 and 80.3±1.64% in control group. No differences were detected the groups

Discussion

The present study confirmed that additional AA is essential for male rainbow trout brood stock. AA affected positively some parameters of sperm quality in rainbow trout, such as sperm concentration, motility and spermatocrit values (Table 2). Rainbow trout males given 300 mg kg^{-1} AA supplemented diet for 3 months showed the best sperm concentration among AA800 and AAC groups. Averages sperm concentration of AA300 and AA800 were found significant when compar ed to the average of AAC group, but no statistical differences were detected between the averages all of experimental groups ($P>0.05$). Although the AA mechanism of how to effect sperm number in animals is not known clearly, there are several researches that reported positive effect of AA on sperm concentration in human and animals. AA concentration in trout seminal plasma is directly affected by dietary supply of vitamin C and also by the spawning season. And AA deficiency significantly reduced the sperm concentration in rainbow trout (Dabrowski and Ciereszko 1995). AA supplementation of human patients caused significant increase in sperm count and sperm volume (Dabrowski and Ciereszko, 2001). AA, AA-vitamin E combination increased sperm concentration and total sperm output in male rabbit (Yousef *et al.*, 2003). Unlike this, lack of vitamin C supplementation in Guiana pigs and humans was resulted in low sperm count (Chinoy *et al*., 1986; Dawson *et al*., 1992).

Average spermatocrit values were also found higher in AA experiment groups than control group. The highest spermatocrit was measured in AA800 group and average of both AA300 and AA800 was found significant when compared to average of AAC group ($P<0.05$). On the other hand, spermatocrit of AA800 was measured higher than AA300 and there was no significance detected. Spermatocrit is directly related to sperm concentration, so spermatocrit values fit into sperm concentrations of groups respectively. A direct relationship between sperm density and spermatocrit of fish sperm has been established in coho salmon, *Oncorhynchus kisutch* (Bouck and

Table 2. Sperm quality parameters obtained from experimental groups and control groups

	AA300	AA800	AAC
Spermatozoa concentration (cell ml^{-1})	$11.88\ x10^9 \pm 0.335\ x10^{6\ a}$	$11.75\ x10^9 \pm 0.35\ x10^{6\ a}$	$9.875x10^9 \pm 0.23x10^{6\ b}$
Spermatocrit (%)	23.6±0.95[a]	25.80±0.99[a]	20.1±1.25[b]
Motility duration (Sec.)	38.4±1.58[a]	36.9±0.85[a]	35.4±1.6[a]
Motility (%)	81.19±2.45[a]	88.99±1.07[b]	78.25±3.63[a]
Fertilization ability	83.5±2.16[a]	87.8±2.05[b]	80.3±1.64[a]

AA300: group was feed with 300 mg/kg ascorbic acid supplemented diet; AA800: Group was feed with 800 mg/kg AA supplemented diet; AAC: Group was feed with non ascorbic acid supplemented diet. In same cases, means with the different letter differs at $P<0.05$.

Jacobson, 1976), Atlantic salmon, *Salmo salar* (Piironen and Hyvärinen, 1983), Atlantic halibut, *Hippoglossus hippoglossus* (Tvedt *et al*., 2001).

Motility increased with AA supplementation. However only motility of AA800 group was found significant (Table 2). Although motility duration of AA supplemented groups (AA300 and AA800) were found higher than control group, no statistic significance was detected between average of experimental groups and control group ($P>0.05$). It is expected that and percentage of motility and motility duration of AA experiment groups could be found higher than control, because AA protects sperm cells against oxidative damage of free oxygen radicals. The antioxidant function of AA provides a protection for the sperm cells by reducing the risk of lipid peroxidation and AA deficiency reduces both sperm concentration and motility and consequently the fertility (Ciereszko and Dabrowski, 1995). Effect of dietary AA on motility and fertilizing ability of rainbow trout sperm was assayed by Ciereszko and Dabrowski (2000). Sperm was obtained from male fed with 870 mg kg^{-1} AA, stored 14 days. And after cool storage, vitamin C supplemented group's sperm showed higher motility than non supplemented group. Dabrowski and Ciereszko (1996) reported that when the seminal plasma AA concentration decrease sperm quality is negatively influenced in rainbow trout.

Male rainbow trout given diets supplemented with 800 mg kg^{-1} AA had higher percentage of fertilizing ability than the dose of 300 mg kg^{-1} and non AA supplemented group and average of AA800 was found significant when compared to AA300 and AAC. Eyed embryo found higher in groups fed with 300 mg kg^{-1} AA than control group fed without AA supplemented diet, but differences between averages were not statistically significant. Similarly, Ciereszko and Dabrowski (2000) reported that higher fertilizing ability in rainbow trout milt was obtained in group supplemented with 870 mg kg^{-1} vitamin C after 14 days of storage.

Results of this study showed that additional AA supplementation useful for improving sperm quality of rainbow trout. The antioxidant capacity of AA effects sperm concentration, motility and fertilizing ability positively. Protective role of AA on male germ cells are known, but later researches may be design to explain how AA increase sperm concentration in male rainbow trout.

Acknowledgements

This study was supported by a grant from Ege University Scientific Researches Projects Fund, İzmir, Turkiye. (Project number: 03-BIL-003). We would like to thank managers of the private hatcheries for their support and cooperation.

References

Benitez, L.V. and Halver, J.E. 1982. Ascorbic acid sulfate sulfohydrolase (C_2 sulfatase): The modulator of cellular levels of L-ascorbic acid in rainbow trout. Proc. Natl. Acad. Sci., 79: 5445-5449.

Bouck, G.R. and Jacobson, J. 1976. Estimation of salmonid sperm concentration by microhematocrit technique. Trans. Am. Fish. Soc., 105: 534–535.

Bromage, N.R. 1995. Broodstock management and seed quality-general considerations. In: N.R. Bromage and R.J Roberts (Eds.), Broodstock management and egg and larval quality. Blackwell Science Ltd., Oxford: 1-24.

Ciereszko, A. and Dabrowski, K. 1995. Sperm quality and ascorbic acid concentration in rainbow trout semen are affected by dietary vitamin C: An across-season study. Biol. Reprod., 52: 982-988.

Ciereszko, A., Liu, L. and Dabrowski, K. 1996. Effect of season and dietary ascorbic acid on some biochemical characteristics of rainbow trout (*Oncohrynchus mykiss*) semen. Fish Physiol. Biochem., 15: 1-10.

Ciereszko, A., Dabrowski, K., Lin, F. and Liu, L. 1999. Protective role of ascorbic acid against damage to male germ cells in rainbow trout (*Oncohrynchus mykiss*). Can. J. Fish. Aquat. Sci., 56: 178-183.

Ciereszko, A. and Dabrowski, K. 2000. Effect of ascorbic acid supplement *in vitro* on rainbow trout sperm viability. Aquaculture Int., 8: 1-8.

Chinoy, N.J., Mehta, R.R., Seethalakshmi, L., Sharma, J.D. and Chinoy, M.R. 1986. Effects of vitamin C deficiency on physiology of male reproductive organs of guinea pigs. Int. J. Fertil., 31: 232-239.

Dabrowski, K. and Ciereszko, A. 1995. Protective effect of seminal plasma proteins on the degradation of ascorbic acid. Mol. Cell. Biochem., 148: 59-66.

Dabrowski, K. and Ciereszko, A. 1996. Ascorbic acid protects against male infertility in a teleost fish. Experientia, 52: 97-100.

Dabrowski, K. and Ciereszko, A. 2001. Ascorbic acid and reproduction in fish: endocrine regulation and gamete quality. Aquaculture Res., 32: 623-638.

Dawson, E.B., Harris, W.A., Tetter, M.C. and Powell, L.C. 1992. Effect of ascorbic acid supplementation on sperm quality of smokers. Fertil. Steril., 58: 1034-1039.

Fraga, C.G., Motchnick, P.A., Shigenaga, M.K., Helbock, H.J., Jacob, R.A. and Ames, B.N. 1991. Ascorbic acid protect against endogenous oxidative DNA damage to human sperm. Proc. Natl. Acad. Sci., 88: 11003-11006.

Halver, J.E., Ashley, L.M. and Smith, M. 1969. Ascorbic acid requirements of coho salmon and rainbow trout. Trans. Am. Fish Soc., 98: 762-771.

Hilton, J.W., Cho, C.Y. and Slinger, S.L. 1978. Effect of graded levels of supplemental ascorbic acid in practical trout diets fed to rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can., 35: 431-436.

Izquierdo, M.S., Fernandez-Palácios, H. and Tacon, A.G.J. 2001: Effect of broodstock nutrition on reproductive performance of fish. Aquaculture, 197: 25-42.

Johnston, W.L., MacDonald, E. and Hilton, J.W. 1989. Relationships between dietary ascorbic acid status and deficiency, weight gain and brain neurotransmitter levels in juvenile rainbow trout, *Salmo gairdneri.* Fish Physiol. Biochem., 6(6): 353-365.

Konuk, T. 1975. Practical Physiology. Ankara University Veterinary Medicine Publication, Ankara University Press, Ankara, 250 pp.

Lee, K.J. and Dabrowski, K. 2004. Long-term effects and interactions of dietary vitamins C and E on growth

and reproduction of yellow perch, *Perca flavescens*. Aquaculture, 230: 377-389.

Liu, L., Dabrowski, K. and Ciereszko, A. 1995. Protective effect of seminal plasma proteins on the degradation of ascorbic acid. Mol. Cell. Biochem., 148: 59-66.

Liu, L., Ciereszko, A. and Dabrowski, K. 1997. Dietary ascorbyl monophosphat depress lipid peroxidation in rainbow trout spermatozoa. J. Aquat. Anim. Health., 9: 249-257.

Lovell, R.T. and Lim. C. 1978. Vitamin C in pond diets for channel catfish. Trans. Am. Fish. Soc., 107: 321-325.

Piironen, J. and Hyvärinen, H. 1983. Composition of the milt of some teleost fishes. J. Fish Biol., 22: 351–361.

Sandnes, K., Torrissen, O. and Waagbø, R. 1992. The minimum dietary requirement of vitamin C in Atlantic salmon (*Salmo salar*) fry using Ca ascorbate-2-monophosphate as dietary source. Fish Physiol. Biochem., 10(4): 315-319.

Sato, M., Yoshinake, R. and Ikeda, S. 1978. Dietary ascorbic acid requirement of rainbow trout for growth and collagen formation. Bull. Jpn. Soc. Sci. Fish., 44: 1029-1044.

Sinclair, S. 2000: Male infertility: nutritional and environmental considerations. Alternatives Med. Rev., 5: 28–38.

Tvedt, H.B., Benfey, T.J., Martin-Robichaud, D.J. and Power, J. 2001. The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture, 194: 191-200.

Yousef, M.I., Abdallah, G.A. and Kamel, K.I. 2003. Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits. Anim. Reprod. Sci., 76: 99-111.

Waagbø, R. and Sandnes, K. 1996. Effects of dietary vitamin C on growth and parr-smolt transformation in Atlantic salmon, *Salmo salar* L. Aquaculture Nutr., 2(2): 65-69.