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Research Articles

Ascorbic acid protects against male infertility in a teleost fish

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Abstract. An animal unable to synthesize ascorbic acid uniquely mimicks human and non-human primates. Therefore, in this study we used the rainbow trout, a teleost fish, as the model animal to study the importance of dietary ascorbic acid on the fertilizing ability of sperm. A high concentration of ascorbic acid in semen plays a key role in maintaining the genetic integrity of sperm cells, by preventing oxidative damage to sperm DNA. This study will show that the concentration of ascorbic acid in seminal plasma reflects the dietary intake of vitamin C. The concentration of ascorbic acid in seminal plasma of fish declined significantly in groups fed either an ascorbate-free diet (from 4.74 ± 0.9 to 0.16 ± 0.08 μg ml^{-1}) or an ascorbate-rich diet (from 37.9 ± 4.7 to 17.7 ± 3.2 μg ml^{-1}) during the spermiation season. The relationship between ascorbate status and fertility was studied in six groups of fish fed graded levels of ascorbic acid, which spermiated over a 150-day-period. Sperm from individual males was used to fertilize several batches of eggs. When the seminal plasma ascorbate concentration decreased to 7.3 μg ml^{-1} a significant decrease of fertilization rate and the hatching rate of embryos resulted. This is the first evidence that dietary ascorbate level directly affected sperm quality and influenced male fertility in a scurvy-prone vertebrate.
Key words. Vitamin C; spermatozoa; ascorbate-deficient; salmonid.

The rainbow trout (*Oncorhynchus mykiss*) was used as a model animal to study the biological consequences of ascorbic acid deficiency on male fertility. It is a useful model because of its mode of reproduction, high fecundity of females, external fertilization and embryonic development, and ease of sperm manipulation[1]. Some aspects of the role of ascorbic acid in humans can be studied in mammalian models, such as mice and rats, and large domestic animals. However, teleost fish such as the rainbow trout are valuable to answer specific scientific questions related to the essentially of ascorbic acid in humans and primates because these fish are also unable to synthesize ascorbic acid[2,3], whereas the above-mentioned mammals can.

In a study of male smokers, a daily 200 or 100 mg day^{-1} dietary supplementation with ascorbic acid increased sperm motility[4–6], and in comparison to placebo group increased viability and decreased sperm agglutination. Scorbutic conditions in humans result in a microscopically measurable increase in sperm agglutination, whereas dietary ascorbate supplementation enhances male fertility and increases pregnancy rates[6]. The protective function of ascorbate in human sperm as an antioxidant, which prevents oxidative damage to sperm DNA, has recently been addressed by Fraga et al[7–8]. High concentrations of ascorbate in seminal plasma are a defense against free radicals which may otherwise produce the oxidized nucleotide(s) (8-oxo-7,8-dihydro-2′ deoxyguanosine) which are responsible for mutations during DNA replication subsequently leading to heritable mutations and birth defects. Oxidative damage to sperm DNA has not been associated with sperm viability or motility, therefore mutations of paternal origin are transmitted at a higher frequency than those from the maternal germline[9].

Materials and methods

Two-year-old rainbow trout (golden variety of London, Ohio, strain) were maintained at a density of 11 fish (mean male weight 245 ± 85 g, $n = 30$) per tank in 750 l indoor fiberglass tanks, each supplied with a constant flow of well water (10–14 °C). The fish in groups 1–6 (5–8 males per group) were fed twice daily at the beginning of the trial (1% body weight per day) on fish meal-based diets supplemented with 0, 30, 110, 220, 440 and 870 ppm of ascorbyl monophosphate (AP) magnesium salt (Showa Denko America Co., New York, NY, U.S.A.), respectively. The level of ascorbic acid in this preparation was 43.3%. The experiment began on May 14, 1992, and the body weight gain (133 ± 53, 111 ± 18, 149 ± 44, 104 ± 19, 112 ± 61 and 173 ± 49% for groups 1–6, respectively) did not differ significantly ($p < 0.05$, Scheffe's test) at the beginning of spermiation (September 28, 1992). The first milt (spermatozoa + seminal plasma) was collected on October 26, 1992 (day 1 in figs 1 and 2). Fish were anesthetized in MS 222 (Argent

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Chem., Washington) and milt was then stripped from males by gentle abdominal massage[10–12] and collected in a plastic container, weighed, and kept on ice (0 °C) until analysis and fertilization (6–8 h). Milt was centrifuged and seminal plasma mixed with protein precipitating solution containing 0.8% EDTA to analyze the total ascorbic acid (reduced and oxidized form)[13–15]. Earlier studies had shown the excellent bioavailability of ascorbyl monophosphate in teleost fish[16–20].

We used a spectrophotometric technique for estimating spermatozoa counts in individual males[21]. In vitro fertilization was used to test sperm viability. Two strains of rainbow trout were used as the egg donors; the London, Ohio strain, (October–December) females, and spring-spawners, the Mt. Shasta, California strain (Januar–March). To fertilize a batch of 100–250 eggs taken either from a single female (days 10 and 25) or 3-4 females (days 44-144), the sperm density of individual males was counted, and to avoid an excess of sperm, which may mask viability, we tested the marginal density using procedures previously described[10–12]. In subsequent fertilizations the sperm densities used were: day 10, 1×10^5/egg; day 25, 1×10^6/egg; day 74, 1×10^6/egg; day 116, 0.5×10^5/egg; and day 144, 2.5×10^5/egg. The survival of embryos was determined at the 'eyed stage', when intensive pigementation of the embryo's eyes is noticeable through the egg's external shell. At this stage, embryos become less sensitive to manipulation, and dead eggs can be removed. The hatching rate was the second criterion used to determine viability of the offspring. The mortality which may occur between these two events has been related to stress during maturation. Alevins from individual males were raised in 30 l tanks with continuous water flow (10 °C) and fed a starter diet (Bioproducts, Oregon). Relationship between the hatching embryos (HE) and the seminal plasma total ascorbate concentration (AA) was calculated as an exponential rise to maximum according to the equation: $AA = a \text{ Log } (HE) + b$.

Results

Male rainbow trout spermiate for an extended period[10–12], and observations of dietary effects on the ascorbate status can be monitored in their seminal plasma. This effect is exacerbated during spermatogenesis in fish devoid of dietary ascorbate. The effects of marginal dietary levels can also be determined (fig. 1). There were no significant differences ($p < 0.05$) in somatic weight between the groups at the end of the experiment. This is in accordance with previous findings that demonstrated that body weight needs to increase 8–10 fold before growth depression and signs of scurvy become apparent in teleost fish[22–24]. Thus the response of the seminal plasma ascorbate, rather than body weight, should be considered as a measure of the dietary requirement of vitamin C for male fertility optimization. Our results demonstrate that despite the absence of significant differences in weight gain, the differences in ascorbate concentration of seminal plasma during the permiation season were substantial and correlated with dietary treatments.

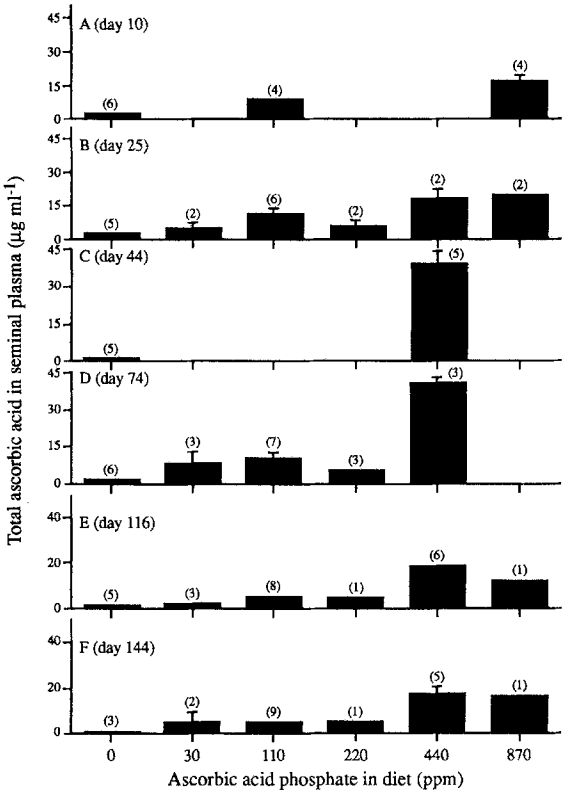

Figure 1. The relation between the dietary level of ascorbate (expressed as pp. of monophosphate ester of ascorbic acid) and total ascorbic acid concentration in seminal plasma. Data are missing for days and groups in which no spermiating males were identified. The day (10–144) indicates the sampling of sperm from the beginning of the spermiation period (September 28, 1992). Numbers in parentheses refer to males examined.

The concentrations of total ascorbate in seminal plasma declined significantly ($p < 0.01$) during the spermiation season both in group 1, fed an ascorbate-free diet, (from 4.74 ± 0.9 to 0.16 ± 0.08 µg ml^{-1}) in group 5, with a 440 ppm supplemented diet (from 37.9 ± 4.7 µg · ml^{-1} (day 44) to 17.7 ± 3.2 µg ml^{-1} (day 144)) (fig. 1). Significant differences in ascorbate concentrations between dietary treatments were found ($p < 0.01$) on days 10 and 25, and on other days ($p < 0.05$). The presence of a high ascorbate levels in plasma were concident with high percentages of the reduced form (80–90%) whereas with low ascorbate a large proportion occurred in the dehydroascorbate form (data not shown). Thus, the effects of reduced ascorbate as an antioxidant would be magnified at high seminal plasma levels. Conversely, dehydroascorbic acid is Na-independently transported across membranes and has been shown to have adverse effects on cell membranes[25–27]. In view of this result we hypothesized that reduced

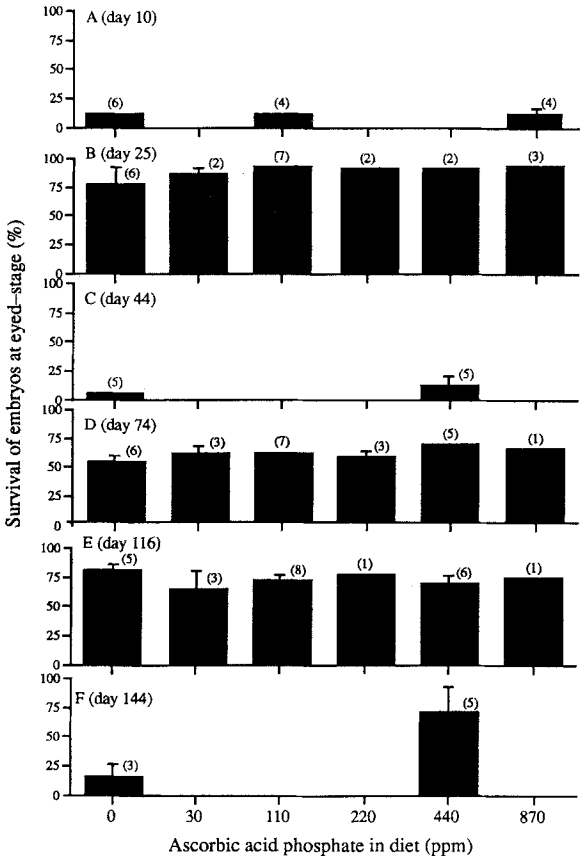

Figure 2. The effect of dietary treatment (ascorbic monophosphate level 0–870 ppm) on the survival of embryos until the eyed stage (externally recognizable black pigment in embryos; 20–22 days of incubation of 10 °C). The means were significantly different ($p < 0.05$) on two occasions (C and F). In the last trial we irradiated sperm with 6 W/m^2 during 10 min and only in this case was the difference significant. The number in parentheses refers to the number of males analyzed.

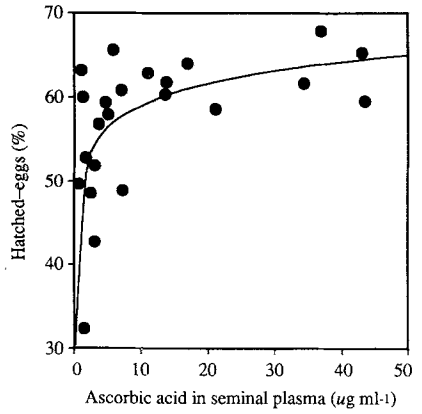

Figure 3. The relation between total ascorbate concentration in the seminal plasma (AA) from individual males and their respective fertilization rate (expressed as percent of 'hatched embryos' (HE)). Data are presented for day 74 (D); however, significant correlations were found between AA and EE (%) for the following experiments: day 44; LnAA = 0.264 HE − 0.824; r = 0.739; $p < 0.05$; day 74; LnAA = 0.079 HE − 2.858; r = 0.541; $p < 0.01$ (see fig. 2).

seminal plasma antioxidant status would cause a dramatic change in sperm cell integrity, increase abnormalities, and reduce fertilization rate.

To investigate sperm viability we used in vitro fertilization and tested individual males against either one female or batches of females (fig. 2). The fact that we tested a marginal sperm density, to avoid masking the effect of sperm viability, resulted in an increased variation of embryo survival from day to day. In two out of six experiments (day 44 and 144) the survival of embryos to the 'eyed stage' was reduced by the ascorbate status of the males. On day 74 (experiment D), the differences between the groups were not significant. However, the analysis of all examined males revealed a correlation between the hatching rate (HE) and the seminal plasma concentration of ascorbate, in accordance with the exponential rise to a maximum (AA = 8.57 Log HE + 50.44) (fig. 3). Calculations of the threshold for this relationship (90% of the HE maximum value) indicated that when seminal plasma ascorbate concentration was lower than 7.3 μg ml^{-1}, survival of embryos from those males was depressed. Embryos from the 74 day experiment were raised under standard conditions and no differences in growth rate or percentage of abnormal fish were observed until day 90 after hatching (data not shown).

Discussion

Males were able to minimize the effects of dietary ascorbate omission for a prolonged period (5 months). Seminal plasma levels of ascorbate in October were fairly high compared to the blood plasma levels in fish devoid of ascorbate for this extended period[16,17,20,23,27]. As suggested in the case of human sperm[7–8], high ascorbate concentrations in seminal plasma might play a protective role against oxidatie DNA damage during fish spermatogenesis and durig storage of inactive spermatozoa[102] in seminiferous tubules. Oxidative damage to sperm DNA, resulting in mutations, is likely to occur during the early stages of spermatogenesis[7,8]. Therefore, the observed effect in the present study might have occurred earlier, so that paternally inherited mutations were observed. Scorbutic conditions in male reproduction were easily reversed by dietary saturation with ascorbic acid[4–6]. However, the presence of defect producing mutations derived from a paternal germline will remain a risk factor for an extended period.

The damage to fish spermatozoa reported here was sufficiently extensive to influence viability. The protective role of ascorbate in the maturation process of sperm may be particularly critical in situations where cryopreservation is used, or as a result of environmental disturbances (stress or intoxication)[29–30]. This study

illustrates the use of a fish biomodel to study reproductive toxicology. In its inability to synthesize ascorbic acid, this model resembles humans and primates. The benefit of an ascorbate dietary level in excess of 440 ppm AP might be able to offset the effects of environmental stress on sperm quality.

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