

## Short Paper

# Effects of vitamin C on testicular and seminal characteristics of Markhoz goats

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## Summary

The effect of subcutaneous injections of vitamin C on the seminal characteristics of Markhoz bucks (2–4-year-old) was studied. The bucks, trained to serve an artificial vagina, were randomly allotted into three equal groups (n = 4) and received daily either zero (1 mL normal saline; control group), or 20 (VitC20 group) or 40 (VitC40 group) mg per kg body weight vitamin C from July 06, 2006 to Oct. 06, 2006. Blood samples were taken from the jugular vein at monthly intervals, and semen samples were collected at 15-day intervals. Testicular dimensions in the scrotum (circumference, width, and length) were also determined on the day before semen collection. The ejaculates were evaluated for volume, sperm concentration, pH, motility, and abnormal and live sperm. Testicular measurements were not affected by administration of vitamin C. The interaction between vitamin C and the sampling time was significant (P<0.05) for the concentration of vitamin C in the blood plasma and seminal fluid, sperm motility, sperm viability, sperm abnormality, and the number of live-normal sperm in the ejaculate. Vitamin C increased the levels of vitamin C in blood and seminal plasma. Both doses of vitamin C increased the percentage of progressively motile sperm showing forward motility. VitC40 injection for 90 days increased sperm motility and the effect was still evident up to 30 days after the cessation of injections. The percentage of live sperm and mass motility showed similar trends. Both doses were equally effective in decreasing the percentage of abnormal sperm. The total number of live and normal sperm in the ejaculate increased by vitamin C injections and the effect was still evident after the injections had been discontinued. The present data indicates the importance of vitamin C in the reproduction of male goats, as also shown for several mammalian species. They further show that under certain conditions, the *in vivo* synthesis of this vitamin in ruminants might not be sufficient for optimum reproduction.

**Key words:** Goat, Semen, Sperm, Testis, Vitamin C

## Introduction

Vitamin C (ascorbic acid) has been associated with fertility in primates and it may have evolutionary significance (Millar, 1992). It has also been shown to be important for reproduction in several other mammalian species (Luck *et al.*, 1995). Low or deficient ascorbate levels in human have been associated with low sperm counts, increased number of abnormal sperm, reduced fertility, and agglutination (Wilson, 1954; Harris *et al.*, 1979; Dawson *et al.*,

1990). Earlier works showed a beneficial effect of subcutaneous injections of ascorbic acid to sub-fertile bulls (Phillips *et al.*, 1940), cows (Phillips *et al.*, 1941) and stallions (Ralston *et al.*, 1988). Ascorbic acid supplementation of sub-fertile bulls increased its plasma and seminal concentrations (Phillips *et al.*, 1940). These findings indicate that the *in vivo* production of vitamin C in some individuals of the species not dependent on their dietary vitamin C content, may not be sufficiently large enough to support some of the

physiological processes. Tomar *et al.* (1979) reported that the addition of ascorbic acid to liquid extenders for bull semen resulted in an increase in the fertility of cows inseminated with such semen. Addition of 25 mM ascorbic acid to the semen diluent for Murrah buffalo resulted in a significantly higher post-thaw sperm motility, and percentage of live spermatozoa as compared with the untreated diluent (Sing *et al.*, 1996). The small beneficial effect of feeding of 1 g per day of ascorbic acid reported in rams (Asghari, 1999) and goats (Heidari, 2002) during the breeding season might be due to denaturing of this vitamin in the rumen (Black and Hidiroglou, 1996; Hidiroglou *et al.*, 1997). On the other hand, Sonmez and Demirci (2003) reported that intramuscular injection of 20 mg ascorbic acid per kg live weight for 30 days increased semen volume, sperm concentration and motility of rams, and also increased its concentration in blood and seminal plasma. The effect of supplemental vitamin C on the parameters of reproduction in fertile male goats has not been reported; therefore, an experiment was conducted to investigate the effect of a daily subcutaneous injection of ascorbic acid on semen characteristics of Markhoz goats. The injection started some time before the natural increase in sperm production, which is highest during summer and autumn (Talebi *et al.*, 2009). According to Farshad *et al.* (2008), the estrous activity of Markhoz does starts late in summer and peaks in November.

## Materials and Methods

Twelve healthy and fertile Iranian native Markhoz bucks (2–4-year-old and weighing 38 to 66 kg) belonging to Markhoz Goat Research Center, near Sanandaj in the west of Iran, were used in this study. During the experiment, the bucks were housed separately from the does and received a daily ration consisting of (as fed) 670 g alfalfa hay and 780 g of a commercial concentrate. The ration contained (dry matter basis) 2.15 Mcal metabolizable energy per kg, 14.0% crude protein, 1.04% calcium and 0.5% phosphorus. The bucks were randomly allotted into three groups (4 animals each) and received subcutaneous

injections of either 0 (VitC0), 20 (VitC20) or 40 (VitC40) mg vitamin C per kg live weight per day for 90 days (July 6, 2006 to October 6, 2006). The bucks were weighed monthly and the ascorbic acid dosage was adjusted accordingly. The experiment was started on June 15, and data were collected from July 6, to October 6, 2006. Vitamin C (L-ascorbic acid, Merck, 64271 Darmstadt, Germany) was dissolved in double distilled water and filtered through a 25 µm filter before injection. The control animals were injected with 0.9% sodium chloride solution.

Semen collection (Evans and Maxwell, 1989) was performed one day prior to starting ascorbic acid injections, 30 days after the first injection and again at 15-day intervals on 8 occasions. Semen samples were transferred at 35 to 37°C within 15 min of collection and kept in a water bath at 35°C, and seminal parameters (volume, pH, sperm concentration, overall sperm motility, percent of motile sperm, percent of sperm showing forward motility, percent of live sperm, percent of sperm showing morphological abnormalities) were evaluated (Evans and Maxwell, 1989). Seminal pH was measured immediately using a digital pH meter (pH 211 Microprocessor, Hanna, Italy). Overall sperm motility (wave motion) was evaluated on a scale of 0 (all spermatozoa motionless) to 5 (90% or more of spermatozoa active) at a magnification of ×100 (Evans and Maxwell, 1989). Sperm concentration was determined by Neubaur hemocytometer, diluting a small drop of the semen with 2% eosin solution (1:200). The percentages of motile and progressively motile sperm were determined microscopically (×400) by placing a drop of diluted semen (1:20) on a warm slide (35°C) and observing 300 sperm. The semen was diluted in a diluent containing Tris (3.78 g), citrate (2.17 g) and fructose (1.0 g) in 100 mL distilled water. The percentages of live sperm and abnormal sperm were determined by observing 300 spermatozoa, after eosin-nigrosin staining, using a light microscope at ×400 magnification (Evans and Maxwell, 1989). Immediately after semen evaluation, the seminal fluid was separated by centrifugation at 10000 rpm for 30 min, and seminal fluid samples were kept at -20°C

until analyzed for vitamin C concentration. To study the effect of vitamin C administration on its blood plasma concentration, jugular vein blood samples were drawn into EDTA-containing vacutainers one day before injection started, and again at monthly intervals. Blood samples were transferred to the laboratory on ice, centrifuged at 3000 rpm for 15 min, and the plasma samples were stored at  $-60^{\circ}\text{C}$  until analysis. Vitamin C concentration in the seminal fluid and blood plasma was measured according to Kleszczewski and Kleszczewska (2001). Testicular measurements were made on each buck at monthly intervals. Scrotal circumference was measured by using a tape measure, and the length and width were determined by using a caliper.

Statistical analysis of the data was performed by the Proc Mixed of the SAS (1996) for the repeated measure data using a model containing the effects of vitamin C injection, time of sampling and their interaction. The age and weight of the animals were used as the covariates. The percentage data were arcsine-transformed before analysis. The means were compared by the least squares means adjusted for Tukey. The significant level was set at  $P \leq 0.05$ .

## Results

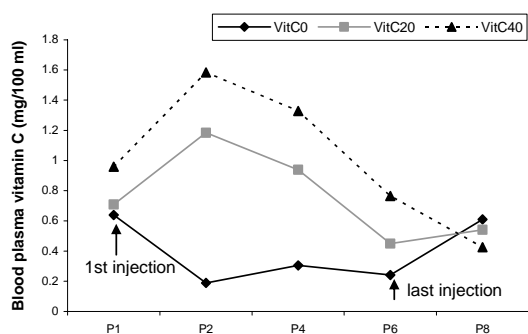
The main effect (Table 1) of vitamin C, and vitamin C by time interaction were not significant for the seminal volume, seminal plasma pH, sperm concentration, total sperm number in the semen sample (overall and

live) and testicular measurements. Analysis of variance showed that vitamin C injection had a significant ( $P < 0.05$ ) interaction with the time of sampling for the concentration of vitamin C in the blood plasma (Fig. 1), seminal plasma (Fig. 2), sperm viability (Fig. 3), sperm abnormality (Fig. 4), and total number of live-normal sperm in the ejaculate (Fig. 5). Initially, vitamin C injection resulted in a sharp increase in the concentration of vitamin C in the blood plasma (Fig. 1) and seminal fluid (Fig. 2), but ascorbic acid concentration started to decrease after 30 days of injection, approaching the control values at 30 days after cessation of injections. The correlation coefficient between the blood and seminal plasma levels of vitamin C was  $-0.03$  ( $P > 0.05$ ),  $0.69$  ( $P < 0.001$ ) and  $0.77$  ( $P < 0.001$ ) for VitC0, VitC20 and VitC40 groups, respectively. Both VitC20 and VitC40 increased the percentage of progressively motile sperm showing forward motility (Fig. 3), but the effect of VitC40 became evident sooner than for VitC20. VitC40 injection for 90 days resulted in an increase of about 15% in sperm motility and the effect was still evident up to 30 days after the cessation of injections. The percentage of live sperm and mass motility showed similar trends (data not shown). VitC20 and VitC40 treatments were equally effective in decreasing the percentage of abnormal sperm in the semen (Fig. 4). There were no significant differences between percentages of abnormal sperm in the vitamin C groups. The percentage of abnormal sperm during the second part of the experimental period was about 6

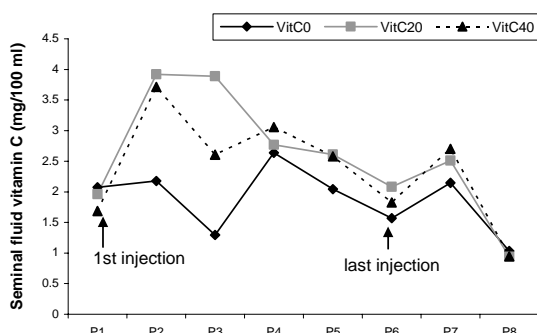
**Table 1: The main effect of vitamin C<sup>†</sup> on goat testis and seminal characteristics (least squares means  $\pm$  SEM)**

Measurements	VitC0	VitC20	VitC40
Scrotal circumference (cm)	27.5 $\pm$ 1.5	27.6 $\pm$ 1.5	29.7 $\pm$ 1.4
Combined testis width in scrotum (cm)	10.5 $\pm$ 1.3	10.5 $\pm$ 1.3	11.4 $\pm$ 1.2
Testis length (cm)	12.6 $\pm$ 0.3	12.8 $\pm$ 0.3	12.3 $\pm$ 0.3
Seminal volume (ml)	1.03 $\pm$ 0.13	1.21 $\pm$ 0.13	1.32 $\pm$ 0.12
Sperm concentration ( $\times 10^9$ /ml)	4.08 $\pm$ 0.40	3.82 $\pm$ 0.39	4.21 $\pm$ 0.37
Seminal pH	6.38 $\pm$ 0.16	6.44 $\pm$ 0.16	6.21 $\pm$ 0.15
Total sperm number in ejaculate ( $\times 10^9$ )	4.25 $\pm$ 0.66	4.62 $\pm$ 0.64	5.56 $\pm$ 0.60
Blood plasma vitamin C level (mg/100 ml)	0.39 $\pm$ 0.08	0.76 $\pm$ 0.07*	1.01 $\pm$ 0.07*
Seminal fluid vitamin C level (mg/100 ml)	1.9 $\pm$ 0.2	2.6 $\pm$ 0.2	2.4 $\pm$ 0.2

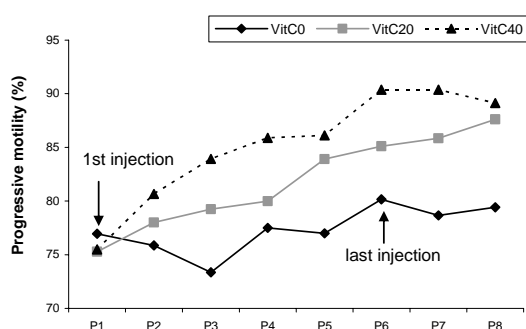
<sup>†</sup>: VitC0, VitC20, and VitC40: 0, 20 and 40 mg vitamin C per kg live weight per day. \*: Significantly higher than the control (VitC0) value ( $P < 0.05$ )



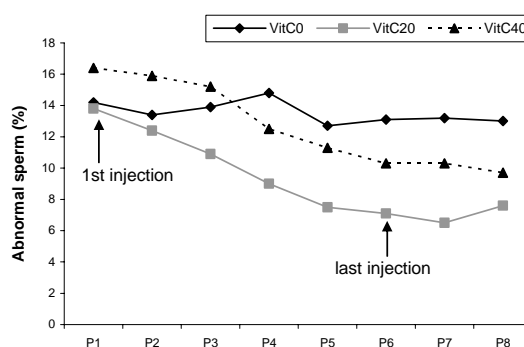
**Fig. 1:** Effect of subcutaneous vitamin C injection on concentration of vitamin C in blood plasma of goats at various periods (P1=Jul. 06, 2006; P8=Nov. 06, 2006) after injection. VitC0, VitC20, and VitC40: 0, 20 and 40 mg vitamin C per kg live weight per day, (SEM=0.13)



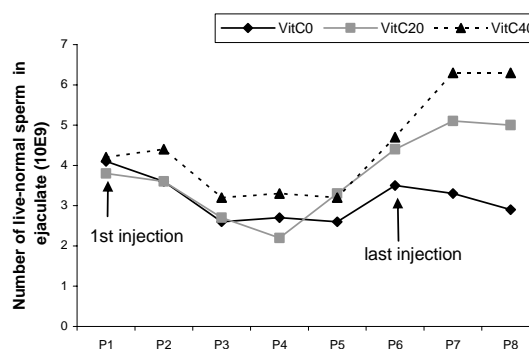
**Fig. 2:** Effect of subcutaneous vitamin C injection on concentration of vitamin C in seminal fluid of goats at various periods (P1=Jul. 06, 2006; P8=Nov. 06, 2006) after injection. VitC0, VitC20, and VitC40: 0, 20 and 40 mg vitamin C per kg live weight per day, (SEM=0.36)



**Fig. 3:** Effect of subcutaneous vitamin C injection on percentage of progressively motile spermatozoa in the semen of goats at various periods (P1=Jul. 06, 2006; P8=Nov. 06, 2006) after injection. VitC0, VitC20, and VitC40: 0, 20 and 40 mg vitamin C per kg live weight per day, (SEM=2.2)



**Fig. 4:** Effect of subcutaneous vitamin C injection on percentage of morphologically abnormal spermatozoa in the semen of goats at various periods (P1=Jul. 06, 2006; P8=Nov. 06, 2006) after injection. VitC0, VitC20, and VitC40: 0, 20 and 40 mg vitamin C per kg live weight per day, (SEM=1.1)



**Fig. 5:** Effect of subcutaneous vitamin C injection on total number of live-normal spermatozoa in the ejaculate of goats at various periods (P1=Jul. 06, 2006; P8=Nov. 06, 2006) after injection. VitC0, VitC20, and VitC40: 0, 20 and 40 mg vitamin C per kg live weight per day, (SEM=0.68)

percentage points lower in vitamin C bucks. The total number of live and normal sperm in the ejaculate also increased by vitamin C injections (Fig. 5) and the effect was still evident after the injections had been discontinued.

## Discussion

Vitamin C is important for reproduction in several mammalian species (Luck *et al.*, 1995) and earlier works showed a beneficial effect of subcutaneous injections of ascorbic acid to sub-fertile bulls (Phillips *et al.*, 1940), cows (Phillips *et al.*, 1941) and stallions (Ralston *et al.*, 1988). Ascorbic acid supplementation of sub-fertile bulls

increased its plasma and seminal concentrations (Phillips *et al.*, 1940) and improved their fertility. However, studies on the effect of supplemental vitamin C on reproductive characteristics of normal male ruminants are scarce. The significant but small beneficial effect of feeding of ascorbic acid to rams (Asghari, 1999) and goats (Heidari, 2002) seems to have resulted from the breakdown of vitamin C in the rumen (Black and Hidiroglou, 1996; Hidiroglou *et al.*, 1997). Subsequently, Sonmez and Demirci (2003) found that the intramuscular injection of ascorbic acid for 30 days increased the semen quality of normal rams and increased its concentration in blood and seminal plasma. Our data support the latter findings; however, we found that the blood and seminal levels of vitamin C declined gradually after reaching a peak within 30 to 45 days of injection. At the same time, there was no significant difference between doses of vitamin C for the level of vitamin C in the seminal fluid. These findings indicate that there might be mechanisms controlling the level of this vitamin in blood and the semen, despite the continued injection of the vitamin. The concentration of vitamin C in the seminal fluid of men was reported to be 8 to 10 times higher than in the blood plasma (Jacob *et al.*, 1992). In Markhoz goats, the ratio of seminal fluid to blood plasma concentrations of vitamin C was 5, 4, and 2.5 for the control, VitC20 and VitC40 groups, respectively; indicating the presence of mechanism (s) for concentrating the vitamin in the semen. The effect of the low dose of vitamin C on sperm motility up to 30 days was similar to the findings of Sonmez and Demirci (2003) who reported about 2% improvement in sperm motility of rams. However, we observed further increases in sperm viability as vitamin C injections continued for longer periods than in their study. The higher dose of vitamin C resulted in greater improvement in sperm viability than the lower dose. On day 90, the percentages of sperm showing forward motility were 80, 85 and 90 for VitC0, VitC20, and VitC40, respectively.

Vitamin C is a strong antioxidant and decreases fatty acid peroxidation in the cell membrane. Vitamin C supplementation decreased free radical formation in human

seminal plasma (Dawson *et al.*, 1992) and decreased the incidence of secondary sperm abnormalities by reducing free radical formation (Sikka, 1996). Vitamin C is also involved in the synthesis of sex steroids such as testosterone, and peptide hormones; hydroxylation of steroids is especially vitamin C-dependent (Luck *et al.*, 1995; Weber *et al.*, 1996). These functions of vitamin C can help improve sperm viability and normality in goats, as found in the present investigation. It seems that at least under certain conditions, such as the present experiment, *in vivo* production of vitamin C may not be sufficient for optimum reproduction in male goats.

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