Effects of Feeding Coenzyme Q10-Ubiquinol on Plasma Coenzyme Q10 Concentrations and Semen Quality in Stallions

Agustin J. Ruiz,[a^] Ahmed Tibary,[a] Robert A. Heaton,[b] Iain P. Hargreaves,[b] Desmond P. Leadon,[c] Warwick M. Bayly[a]*

[a]Department of Veterinary Clinical Sciences, Washington State University, Pullman, WA USA; tibary@wsu.edu;

[b]Liverpool John Moores University, Liverpool, UK; I.P.Hargreaves@ljmu.ac.uk; R.Heaton@2013.ljmu.ac.uk;

[c]Irish Equine Centre, Johnstown, Naas, Co Kildare, Ireland; DLeadon@irishequinecentre.ie;

*Corresponding Author: Warwick Bayly; email: wmb@wsu.edu

^Present address: Newcastle Equine Rehabilitation and Reproduction Center. Newcastle, New South Wales, Australia. agusruizo@gmail.com;

Authors' Contributions: W. Bayly, A. Tibary and D. Leadon conceived the study, A. Tibary, A. Ruiz and W. Bayly designed it, A. Ruiz and A. Tibary acquired the data, R. Heaton and I. Hargreaves assisted with sample analysis, and A. Ruiz, A. Tibary, W. Bayly and D. Leadon interpreted the results and wrote the paper. All authors approved the final manuscript.

Highlights

- 1g ubiquinol/day increased plasma CoQ10 concentrations 2- to 5-fold after 2 weeks
- Plasma CoQ10 concentrations decreased 2 weeks after discontinuing the ubiquinol
- Quality of cooled and frozen semen was enhanced by feeding ubiquinol each day
- Improved semen quality persisted 4 weeks after discontinuing ubiquinol

Abstract

Although coenzyme Q10 (CoQ10) serves as an antioxidant and energy source for spermatozoa when added to stallion semen prior to cooling or freezing, the effects of feeding CoQ10 on semen quality have not been studied. We assessed the effects of daily oral ingestion of CoQ10-ubiquinol by stallions on their plasma CoQ10 concentrations and semen quality.

Seven mature Andalusian stallions ate 1g ubiquinol/day for 4 weeks followed by a 4-week wash-out period. Four horses initially completed an additional 4-week control period without ubiquinol. Blood was sampled weekly for determination of plasma CoQ10 concentrations. Ejaculates were collected every two weeks and assessed for total motility (TM), progressive motility (PM) and viability (V) after cooling for 24h (T_1), immediate cryopreservation (T_2), and cryopreservation following 24h cooling (T_3). Ingesting ubiquinol resulted in an increase in plasma CoQ10 concentration ($p < .001$). Two weeks of CoQ10-ubiquinol resulted in improved V with all treatments (T_1: p=.007; T_2: p=.05; T_3: p=.01) and PM with T_3 (p=.04). In five stallions, TM and PM were also improved for T_1 (p=.01 and p=.02, respectively) and TM increased with T_2 (p=.03). Overall, semen quality parameters increased within the first 2 weeks of supplementation, plateaxued at the end of the 4-week supplementation period and persisted after discontinuing ubiquinol until the end of the sampling period (8 weeks).

Feeding 1g CoQ10-ubiquinol for 4 weeks to breeding stallions improved semen quality after cooling and freezing in 5 of 7 stallions. This could be important for improving reproductive efficiency in stallions.

Keywords: Coenzyme Q_{10}, ubiquinol, antioxidants, plasma CoQ10, semen quality

1 Introduction

Semen quality of stallions is a major factor in determining conception rates in mares [1,2]. Semen quality is affected by several factors, including management, testicular function, age, general health, and provision of enough nutrients to meet the needs of breeding stallions.

Dietary intake of antioxidants, alone or in combination with polyunsaturated fatty acids has been shown to improve semen quality in a variety of species including swine [3, 4], rats [5], humans [6] and poultry [7]. In the stallion, several studies have examined the effect of nutraceuticals in equine diets on sperm quality [8-12]. Among substances with recognized antioxidant effects, coenzyme Q10 (CoQ10; 2,3, dimethoxy-5 methyl-6-decaprenyl benzoquinone) has been shown to prevent effects of oxidative stress on spermatozoa in men [13]. CoQ10 is a naturally occurring fat-soluble vitamin-like substance that plays an important role as a required cofactor in the mitochondrial electron transport chain by serving as an electron shuttle between proton-pumping protein complexes. It is essential for oxidative phosphorylation and aerobic production of adenosine triphosphate (ATP) [14]. Energy for motility and all other energy-dependent processes in spermatozoa depends on the availability of CoQ10 [15,16]. It is also the only naturally synthesized lipid soluble antioxidant present in cellular membranes and circulatory lipoprotein. CoQ10 is found in two forms: ubiquinone (oxidized form) and ubiquinol (fully reduced, aromatic diol form) [17]. Ubiquinol is a potent antioxidant and an effective inhibitor of lipid peroxidation and protein oxidation. Ubiquinol slows down lipid peroxidation due to its ability to assist in the regeneration of antioxidants, such as α-tocopherol and vitamin C. As a result, tissue and plasma concentrations of CoQ10 can become depleted [17].

Ingestion of concentrated CoQ10 powder has been shown to improve semen quality in subfertile/infertile men [13,15,16,18]. In stallions, the addition of CoQ10 to centrifugation extender has been shown to improve post-thaw semen parameters, particularly for stallions with bad semen freezability [19]. However, there is currently no literature describing the effect of eating concentrated CoQ10 on stallion semen quality. Therefore, the aims of this work were to determine the effects of ingestion of a known quantity of CoQ10-ubiquinol by stallions on their plasma COQ10 concentrations and sperm motility and viability after cooling for 24h, cryopreservation soon after collection, and cryopreservation following 24h of cooling, as these treatments currently represent the primary means of preparing and shipping equine semen.

2 Material and methods

The study was approved by the Washington State University Institutional Animal Care and Use Committee. It began in mid-April in the middle of the breeding season in the Pacific Northwest region of the USA and continued until mid-July when the season ends due to extreme heat conditions (average daily maximum ambient temperature > 33°C).

2.1 Horses

Seven client-owned Andalusian stallions (4–20 years of age) of proven fertility and good body condition were used. Stallions were housed in box stalls and had daily exercise in open paddocks. Food and water were provided *ad libitum*. All animals received the same standard diet consisting of a mix of grass hay and alfalfa. In addition to the semen collections associated with this study, each stallion was collected twice weekly for commercial breeding purposes.

All stallions were qualified for inclusion in the study based on the breeding soundness standards set by the American College of Veterinary Theriogenology [20]. Briefly, no abnormalities were found on physical examination, or palpation, measurement and ultrasonography of the testes. Also, two ejaculates were collected one hour apart using a Colorado model artificial vagina with a mounting phantom after 5 days of sexual rest. Each stallion exhibited a normal ejaculatory pattern and had at least 1 x 10^9 progressively motile normal spermatozoa in the second ejaculate.

2.2 Ubiquinol feeding and semen collection schedules

The stallions were randomly assigned to one of two groups. One group was comprised of three stallions. They were started on a daily dietary intake program of 1g of CoQ10-ubiquinol (QHP30, Anlon Nutrition, Kilcullen, Co Kildare, Ireland) mixed into one pound of a grain-based concentrate containing molasses. Ubiquinol was given daily for 4 weeks. This dose was based upon manufacturer's recommendations and a previous study that indicated this dose increased concentrations of CoQ10 in gluteal muscle [21]. In order to facilitate the administration of the correct dose by the owner, gelatin capsules (Torpac, Fairfield, NJ, USA) containing 500mg of ubiquinol were used. The owner broke 2 capsules over the concentrate to provide the desired amount of ubiquinol. Ejaculates were collected every two weeks over an 8 weeks period, for a total of five collections (days 0, 14 and 28 of the feeding period and days 42 and 56 for the post-feeding (wash-out) period) from these three stallions. Jugular venous blood was sampled weekly for measurement of plasma CoQ10 concentrations. The other group of four stallions followed the same regimen with the exception that the period of ubiquinol ingestion was preceded by a four weeks control period with no feeding of ubiquinol powder during which semen was collected every two weeks and plasma CoQ10 concentration was determined each week to monitor their

CoQ10 status while on the control diet. A total of seven ejaculates was collected from these four horses over the entire study period.

2.3 Sample analysis

2.4.1 Plasma CoQ10 concentration

Venous blood was centrifuged, and the plasma removed and frozen until analyzed. Total plasma CoQ10 concentration was determined by HPLC with UV detection at 275 nm [22].

2.4.2 Semen processing and evaluation

Each ejaculate was immediately evaluated for total motility, morphology and concentration of spermatozoa (SpermaCue, Minitube®, Verona, WI, USA), then extended to 50 x 10^6 spermatozoa/ml in a commercial extender (INRA 96, IMV Technologies, L'Aigle, France). Half of the ejaculate was centrifuged at 600 g for 15 minutes. The supernatant was removed, then the sperm pellets were suspended to 200 x 10^6 spermatozoa/ml in a commercial freezing extender (E-Z Freezing "LE"®, Animal Reproduction System Inc, CA, USA), loaded into 0.5 mL straws (IMV Technologies, L'Aigle, France) and frozen in liquid nitrogen vapors (4 cm above liquid nitrogen level) for 20 minutes, then plunged into liquid nitrogen. The other half of the extended semen was cooled in 50 ml plastic tubes (Thermo Fisher Scientific, Waltham, MA, USA) in an Equitainer® (Hamilton-Thorne Biosciences, Beverly, MA, USA) for 24 h. After 24 h of cooling, semen from individual tubes derived from a stallion was pooled. An aliquot of 1 ml of pooled semen was analyzed and the rest of the sample was frozen in liquid nitrogen as described above.

Semen analysis was performed 24 h after cooling (T_1) and after thawing samples frozen both on the farm at 0 h (T_2), and after 24 h of cooling (T_3). Semen was thawed in a water bath at 37°C for

30 secs then diluted one to one (final concentration of 100 x 10^6 spermatozoa/ml) with pre-warmed freezing extender [23].

Semen analysis determined total motility (TM, %) and progressive motility (PM, %) measured by CASA (SpermVision®, Minitube, Verona, WI, USA) with samples loaded on pre-warmed fixed-depth chamber precision slides (20 microns, 3µl; Leja Products BV, The Netherlands) and observed at X200 magnification using the internal calibrations set by the manufacturer. All samples were analyzed after thawing and incubation at 37°C for 10 minutes and verified in a playback mode to make sure that they were consistent, even though the CASA system used did not accept analysis results if the fields analyzed diverged more than 10%. Eight (8) fields were analyzed for each sample. The CASA settings were as follows: 1) frame acquisition = 30 frames/field; 2) sperm with average direction change of < 9.5 µ were considered immotile; and 3) sperm with average curvilinear velocity > 9.5 µ/s, straightness > 0.9 and linearity > 0.5 were considered progressively motile.

Viability (V, %) was determined by SYBR-PI stain at X400 magnification under fluorescent light with a filter of 470nm, in accordance with the manufacturer's protocol (Minitube, Verona, WI, USA). Ten fields were evaluated under fluorescent microscopy (SpermVision®, Mofa®, Verona, WI). At least two hundred cells were counted and classified in two categories: intact – cell stained in green over all its extension; and damaged – nucleus stained in red or nucleus stained in red and the acrosome in green.

2.5 Statistical analysis

The distributions of the plasma concentrations and semen quality parameters were verified with Shapiro-Wilk normality tests. The effect of feeding CoQ10-ubiquinol on plasma concentrations

and semen quality parameters over time was evaluated by 1-way repeated measures ANOVA using commercial analytical software (Statistix 10, Tallahassee, FL, USA). When the F statistic was significant, individual means were compared using the Holm-Sidak method. 2-way ANOVA was used to compare results from the control period with those of the 3 stallions being fed ubiquinol each day over the same period, after individual stallion's values were normalized to reflect percent changes from day 0 results (expressed as 100%) for semen quality parameters measured in ejaculates collected on days 14 and 28. Significance was set at $p \leq 0.05$ in all cases.

3 Results

There was no change in the total plasma CoQ10 concentration of the four stallions during the 4 weeks control period (0.21 ± 0.05 μg/ml). Plasma CoQ10 concentrations increased with daily feeding of ubiquinol ($p < .001$), peaking after 14 days and remaining unchanged for the remainder of the 4 weeks period (Fig 1). Discontinuation of dietary provision of powdered ubiquinol was associated with a gradual decline in plasma CoQ10 concentration. The day 35 concentration was not different to that at the conclusion of the feeding period (day 28) but plasma concentrations on days 42, 49 and 56 were lower than on day 28. Plasma concentrations on days 42, 49 and 56 were not different to those on day 0 of the feeding program or those measured in the four stallions that went through the four weeks control period.

Semen quality did not change for any of the three treatment conditions during the four weeks control period in the four stallions that were sampled over this time (Table 1). After normalizing the day 0 data, there were significant differences when changes in semen quality of the three stallions eating ubiquinol powder each day during this period were compared to those in the four control stallions (Figs 2 – 4).

171 Table 1. Mean values (± SD) for total motility (%), progressive motility (%) and viability (%) in
172 the three semen ejaculates collected from each of 4 control stallions every 2 weeks over a 28 day
173 period. Measurements were made after cooling for 24 hours (T_1), thawing after cryopreservation
174 following collection (T_2), and thawing after cryopreservation that occurred after 24 hours cooling
175 (T_3). Values for each collection time were not different, as reflected by the p values.

Treatment	Semen parameter	Day 0	Day 14	Day 28	p value
T_1	Total Motility %	75.4 ± 17.8	75.9 ± 15.3	77.0 ± 11.2	0.82
T_1	Prog Motility %	68.4 ± 4.3	71.9 ± 6.4	69.4 ± 7.9	0.55
T_1	Viability %	83.7 ± 3.7	85.7 ± 5.8	89.6 ± 9.2	0.23
T_2	Total Motility %	56.0 ± 14.4	53.9 ± 3.6	46.2 ± 10.5	0.36
T_2	Prog Motility %	31.1 ± 17.0	36.9 ± 4.0	37.1 ± 8.7	0.39
T_2	Viability %	35.4 ± 16.8	45.8 ± 7.6	42.6 ± 7.8	0.22
T_3	Total Motility %	23.5 ± 20.5	26.6 ± 24.8	27.7 ± 23.1	0.33
T_3	Prog Motility %	9.6 ± 9.1	16.7 ± 10.8	12.9 ± 9.3	0.14
T_3	Viability %	25.1 ± 14.6	23.2 ± 16.3	22.1 ± 17.2	0.61

176

177 Daily feeding of ubiquinol also resulted in significant improvement in semen quality after 2 and
178 4 weeks when compared to the corresponding values for the day 0 ejaculates (Table 2). Feeding
179 ubiquinol was associated with improved V for T_1, T_2 and T_3. Progressive motility was also
180 improved for T_3 and approached significance with T_1. Results plateaued after 2 weeks of feeding
181 and remained at the same level 2 and 4 weeks after discontinuing the ubiquinol. Results during
182 this wash-out period were better than those on day 0 (i.e., immediately prior to beginning to
183 provide the ubiquinol).

184 Table 2. Total motility, progressive motility and viability of spermatozoa from 7 stallions after
185 24 h cooling (T_1), cryopreservation following collection (T_2) and after cryopreservation

following 24 h cooling (T_3), before (day 0), during (days 14 and 28), and after (days 42 and 56) 4 weeks of daily intake of 1g CoQ10-ubiquinol. Effects of each treatment on semen quality are reflected by p values in the column on the right side. Values with different superscripts are different.

Parameter	Treatment	day 0	day 14	day 28	day 42	day 56	p
Total motility %	T_1	61.9 ± 17.7	78.5 ± 8.1	72.8 ± 10.8	73.9 ± 13.9	73.7 ± 9.3	.10
	T_2	34.8 ± 17.9	44.6 ± 9.9	43.9 ± 12.5	40.7 ± 9.0	39.9 ± 11.5	.35
	T_3	15.7 ± 4.3	26.6 ± 14.0	20.4 ± 10.2	16.0 ± 4.5	23.7 ± 15.4	.14
Progressive motility %	T_1	52.9 ± 20.3	69.6 ± 8.2	63.7 ± 11.2	65.3 ± 15.4	66.8 ± 10.2	.09
	T_2	23.1 ± 14.0	30.2 ± 10.0	28.4 ± 9.9	26.0 ± 7.0	26.9 ± 5.3	.54
	T_3	7.7 ± 6.1^{a}	16.0 ± 10.4^{b}	$11.0 \pm 7.8^{a,b}$	$10.5 \pm 6.2^{a,b}$	11.5 ± 7.8^{b}	.04
Viability %	T_1	68.9 ± 10.5^{a}	82.4 ± 4.9^{b}	78.8 ± 7.5^{b}	81.9 ± 5.6^{b}	82.2 ± 7.4^{b}	.01
	T_2	34.4 ± 13.0^{a}	44.9 ± 7.3^{b}	$41.3 \pm 8.6^{a,b}$	42.4 ± 5.1^{b}	$37.7 \pm 6.3^{a,b}$	.05
	T_3	21.5 ± 6.4^{a}	26.4 ± 9.1^{b}	26.5 ± 9.2^{b}	27.1 ± 6.1^{b}	$26.9 \pm 12.3^{a,b}$	.01

It was noted in the course of evaluating data from individual stallions that two of them consistently demonstrated superior quality semen when compared to the results for the other five stallions. These two stallions showed no improvement in semen quality in response to ubiquinol but amplified the variances in the study population. A second analysis of the data obtained from the other five stallions with lower day 0 results revealed additional positive effects of feeding ubiquinol on TM (p = .01) and PM (p = .02) with T_1 (Fig 5), and on TM (p = .03) but not PM (p = .08) with T_2 (Fig 6), respectively. For T_3, effects of feeding ubiquinol were not significant for TM in the five stallions (Fig 7). This result was not different to that for all seven stallions (Table 2) although the p value was smaller (p = .09). Progressive motility was still improved following T_3 in these five stallions and V was again significantly improved for all 3 semen treatments as was the case when data for all 7 stallions were considered (Table 2).

4 Discussion

The daily inclusion of 1g CoQ10-ubiquinol in the diet of the stallions in this study was associated with a marked increase in the plasma concentration of CoQ10. The average increase was 4- to 5-fold over concentrations recorded on day 0 of the feeding period and in 4 stallions during their 4 weeks control period. The effect of daily feeding of ubiquinol on equine plasma concentrations has not been reported previously. However, the magnitude of the increase in the horses in this study was similar to that reported in people receiving 90mg emulsified ubiquinol daily for 4 weeks [24], and greater than that observed following administration of 800mg ubiquinone for 60 days to six horses [25]. While the bioavailability of ubiquinol and ubiquinone can vary considerably according to the formulation and dose administered [26], most orally ingested CoQ10 is absorbed as ubiquinol, meaning that ubiquinone must first be converted to ubiquinol before being absorbed [27]. Consequently, absorption of powdered ubiquinol is believed to be a more efficient process than absorption of ubiquinone powder, and CoQ10 plasma concentrations are higher following ingestion of ubiquinol [26].

With the exception of the Thoroughbred breeding industry, most equine beeding operations rely heavily on artificial insemination of mares with semen that is either cooled and shipped (equivalent to T_1), frozen soon after collection and transported while frozen (T_2), or initially cooled and then frozen and shipped (T_3). For these reasons we evaluated the effect of feeding ubiquinol to stallions on semen quality evaluated under conditions that were designed to represent the 3 primary ways in which semen is handled and transported. Each of these handling methods stresses the spermatozoa and reduces motility and viability, with initial cooling followed by cryopreservation having the most negative impact. Feeding ubiquinol resulted in

224 significant improvement in viability for all three treatment conditions and in PM for T_3 in all 7
225 stallions. TM was also improved for T_1 and T_2 and PM for T_1 in the five stallions with poorer
226 quality semen when evaluated before the feeding of ubiquinol began. Consequently, it would
227 appear that feeding ubiquinol to stallions each day during the breeding season might help
228 counteract the stress effects of cooling and/or freezing semen and then shipping it. With respect
229 to cooling (T_1), this finding is consistent with the antioxidant properties of CoQ10-ubiquinol
230 inhibiting the formation of hydroperoxides and thus protecting the spermatozoal plasma
231 membrane against oxidation and cold shock [28]. By preventing the oxidation of lipids in
232 spermatozoal membranes, the integrity of the plasma membrane is maintained, and the
233 spermatozoa survive. Similarly, previous studies in humans [28,29], bulls [30], and horses
234 [19,31,32] have shown that CoQ10 acts as an excellent tool in repairing damage to the
235 spermatozoal plasma membrane.

236 The improvement in TM parameters after 24 h cooling (T_1) and cryopreservation shortly after
237 collection of the ejaculate (T_2), and the trend towards improvement with cryopreservation after
238 24 h cooling (T_3) in the subgroup of five stallions, may be associated with a combination of the
239 antioxidative and bioenergetic roles of CoQ10 in the mitochondrial respiratory chain and ATP
240 production in the spermatozoa, especially as cooled and frozen storage of semen are processes
241 known to increase ROS production [33]. Either or both activities may be responsible for the
242 improved semen quality associated with its ingestion in the stallions used in this study.

243 Overall, the semen quality parameters studied were increased 2 weeks after beginning the daily
244 feeding of ubiquinol, reached plateau levels at the end of this 4 week period, and were
245 maintained for the additional 4 weeks after discontinuing the ubiquinol. This was consistent with

the observed increase in plasma concentrations of CoQ10 which peaked two weeks after initiating the feeding of ubiquinol and remained unchanged through the end of the first week after discontinuing it (day 35). A similar rapid increase in CoQ10 concentrations followed by a plateauing of values was reported in equine middle gluteal muscle in response to 3 weeks daily feeding with 1g ubiquinol [21]. Although CoQ10 concentrations in seminal plasma were not determined in the present study, it is possible that the effect of daily ingestion of ubquinol powder on this fluid exhibited a similar pattern. Once a maximum concentration and optimal antioxidative capacity are achieved, further increases might not be possible [34]. Daily oral ingestion of 200 mg ubiquinone has been reported to increase seminal plasma CoQ10 concentration approximately 1.5-fold in men with oligo-, astheno- and teratozoospermia, as well as increasing antioxidative activities of the catalase and super oxide dismutase [35].

Equine gluteal muscle CoQ10 concentrations were not maintained when it was no longer fed [21], which was in contrast to the findings reflecting retention of improved semen quality after stopping provision of ubiquinol in the current study. The retention of improved TM, PM and V after ceasing to feed ubiquinol may indicate increased incorporation of CoQ10 into spermatozoa during the epididymal maturation phase or even during spermatogenesis. Spermatogenesis in the stallion takes approximately 57 days and continues with an epididymal transit of 8 to 11 days [36,37]. During epididymal transit the spermatozoa undergoes several structural and biochemical changes that are critical to its function [36]. The possibility that prolonged improvement in semen quality beyond the period in which ubiquinol was fed was due to either enhancement of sperm structure and function, or improvement of seminal plasma antioxidant activity cannot be ruled out and merits investigation in the future.

Semen quality of stallions can vary during a breeding season, [38] and it is possible that the improvement in semen quality we observed could have reflected this seasonal variation. However, we think that this is unlikely. The study began in mid-April at the height of the breeding season and continued until mid-July at which time it was halted due to previous observations that the intense heat of July and August in central Washington state is accompanied by a drop of semen quality in some stallions. This also prevented us from stretching the post-feeding period another 30 days as it would not have been possible to differentiate between changes in semen quality due to seasonal factors and those that reflected discontinuation of ubiquinol feeding. The duration of a full spermatogenic cycle (~ 60 days) meant that a true crossover study design was not possible; ie, the prolonged period of improved semen quality in response to feeding ubiquinol meant that it was not possible to follow the 4 weeks feeding of ubiquinol with a control period in the 3 stallions in the first treatment group, as this would have coincided with the period when daily ambient temperatures regularly exceed 32°C. However, comparison of the changes in semen quality of the four stallions which served as the control group while the other three were receiving ubiquinol, indicated that the TM, PM and V of the semen of the treated stallions improved significantly while that in the control stallions did not change. When the diet of the initial control group was changed to include ubiquinol powder, the quality of their semen also improved when compared to their control values. Each stallion had the same breeding schedule throughout the period of the study, so it is unlikely that any of the observed improvements in semen quality reflected differences in individual horse's breeding schedules.

The major limitation of our study was the number of stallions used and the duration of the feeding period. There is inherent difficulty in finding sufficient numbers of stallions under the same management at the same period of the reproductive season for studies like these. Stallion semen parameters and the ability to withstand cooling and freezing are highly variable between and within invidual animals, depending on the season and their workload [39,40]. In our study the small number of stallions used and the notable variations between them with respect to semen quality contributed to a large variation of sperm parameters and consequently decreased the power of the study. Inability to implement long-term feeding and correspondingly long washout periods is a common limitation in nutritional studies like the one we have described [8].

Another possible limitation to the study was the use of fluorescent microscopy to determine spermatozoal viability by counting SYBR/PI stained cells. Flow cytometry would have given more precise results, but the equipment was not available. The miscroscopic method is used commonly in practice for assessment of semen quality and with acceptable results in laboratories that do not have flow cytometry capabilities [41]. CASA was used to analyze the semen using internal calibrations set by the manufacturer. CASA analysis generates a large number of variables and the interpretation of results for a number of them can be controversial. For this reason and because, from a practical perspective, total and progressive motility and viability are the parameters most commonly evaluated by equine practitioners, we elected to only report results for these three parameters of semen quality.

Inherent differences in the ability of equine sperm from different stallions to survive cooling and cryopreservation are well known [42]. These individual variations are probably due to differences in sperm biochemistry and metabolism. Differences in membrane composition

(particularly cholesterol concentrations) between stallions results in differences in membrane permeability to water, and cryoprotectants conditioning sperm cryosurvival [43]. It is possible that the individual variations in responses to cooling and freezing were based on differing sensitivities to oxidative stress, and that some stallions benefited more than others from the ingestion of CoQ10-ubiquinol. As demonstrated in one previous study, addition of CoQ10 to the centrifugation extender prior to freezing improved post-thaw sperm parameters most markedly in stallions with poor semen freezing ability [19].

5 Conclusion

In conclusion, the present study provides evidence that daily feeding of 1g CoQ10-ubiquinol increases plasma CoQ10 concentrations and improves sperm quality parameters after cooling and freezing in some stallions. Future research is needed to measure the effect of feeding ubiquinol on CoQ10 concentrations in seminal plasma and to correlate any changes with alterations in semen quality parameters. This is the first such study of the effects of feeding CoQ10-ubiquinol on semen quality. Additional work to evaluate the effects of different daily intakes of ubiquinol on spermatozoal function after cooling and freezing is also indicated in order to maximize fertilization potential of the stallion ejaculate and stallions in breeding programs.

Acknowledgments

Sincere thanks to Ms. Ami MacHugh who generously provided the stallions for this study and assisted in the management of stallions and nutritional supplementation. The help of Dr. Salman Waqas and Dr. Cristian Patino, who assisted with the semen analysis is gratefully acknowledged.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Interest

D. Leadon and W. Bayly are former directors of Anlon Nutrition Ltd, Ireland, which was a small research and development start-up company and supplied the ubiquinol used in this study.

References

[1] Magistrini M, Vidament M, Clement F, Palmer E. Fertility prediction in stallions. Anim Repro Sci 1996;42:181-8.

[2] Parlevliet JM, Colenbrander B. Prediction of first season stallion fertility of 3-year-old Dutch Warmbloods with prebreeding assessment of percentage of morphologically normal live sperm. Equine Vet J 1999;31:248-51.

[3] Strzezek J, Fraser L, Kuklińska M, Dziekońska A, Lecewicz M. Effects of dietary supplementation with polyunsaturated fatty acids and antioxidants on biochemical characteristics of boar semen. Repro Biol 2004;4:271-87.

[4] Audet I, Laforest J-P, Martineau GP, Matte JJ. Effect of vitamin supplements on some aspects of performance, vitamin status, and semen quality in boars. J Anim Sci 2004;82:626-33.

[5] Sönmez M, Türk G, Yüce A. The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. Theriogenology 2005;63:2063-72.

[6] Akmal M, Qadri JQ, Al-Waili NS, Thangal S, Haq A, Saloom KY. Improvement in human semen quality after oral supplementation of vitamin C. J Medicinal Food 2006;9:440-2.

[7] Eid Y, Ebeid T, Younis H. Vitamin E supplementation reduces dexamethasone-induced oxidative stress in chicken semen. Brit Poultry Sci 2006;47:350-6.

[8] Stradaioli G, Sylla L, Zelli R, Chiodi P, Monaci M. Effect of L-carnitine administration on the seminal characteristics of oligoasthenospermic stallions. Theriogenology 2004;62:761-77.

[9] Brinsko SP, Varner DD, Love CC, Blanchard TL, Day BC, Wilson ME. Effect of feeding a DHA-enriched nutriceutical on the quality of fresh, cooled and frozen stallion semen. Theriogenology 2005;63:1519-27.

[10] Deichsel K, Palm F, Koblischke P, Budik S, Aurich C. Effect of a dietary antioxidant supplementation on semen quality in pony stallions. Theriogenology 2008;69:940-5.

[11] Garmsir AK, Shahneh AZ, Jalali SMA, Nouri H, Afshar M. Effects of dietary thyme (Thymus vulgaris) and fish oil on semen quality of miniature Caspian horse. J Equine Vet Sci, 2014;34:1069-75.

[12] Freitas ML, Bouéres CS, Pignataro TA, de Oliveira FJG, Viu MAdO, Oliveira RA. Quality of fresh, cooled, and frozen semen from stallions supplemented with antioxidants and fatty acids J Equine Vet Sci 2016;46:1-6.

[13] Balercia G, Mosca F, Mantero F, Boscaro M, Mancini A, Ricciardo-Lamonica G, et al. Coenzyme q10 supplementation in infertile men with idiopathic asthenozoospermia: an open, uncontrolled pilot study. Fert Sterility 2004;81:93-9.

[14] Hargreaves IP. Ubiquinone:cholesterol`s reclusive cousin. Ann Clin Biochem 2003;40:207-18.

[15] Littarru GP, Tiano L. Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. Molec Biotech 2007;37:31-7.

[16] Lewin A, Lavon H. The effect of coenzyme Q10 on sperm motility and function. Molec Aspects Med 1997;18 S1:213-9.

376 [17] Ernster L, Dallner G. Biochemical, physiological and medical aspects of ubiquinone
377 function. Biochim Biophys Acta - Molec Basis Dis 1997;1271:195-204.

378 [18] Lafuente R, González-Comadrán M, Solà I, López G, Brassesco M, Carreras R, et al.
379 Coenzyme Q10 and male infertility: a meta-analysis. J Assisted Repro Gen 2013;30:1147-56.

380 [19] Carneiro JAM, Canisso IF, Bandeira RS, Scheeren VFC, Freitas-Dell'Aqua CP, Alvarenga
381 MA, et al. Effects of coenzyme Q10 on semen cryopreservation of stallions classified as having
382 good or bad semen freezing ability. Anim Repro Sci 2018;192:107-18.

383 [20] Kenney RM, Hurtgen J, Pierson R, Whiterspoon D, Simons J. Manual for Clinical Fertility
384 Evaluation of the Stallion. Hastings, NE: Society for Theriogenology; 1983.

385 [21] Thueson E, Leadon DP, Heaton R, Hargreaves I, Bayly WM. Effect of daily
386 supplementation with ubiquinol on muscle CoQ10 concentrations. Comp Exercise Phys
387 2019;15:219-26.

388 [22] Duncan AJ, Heales SJ, Mills K, Eaton S, Land JM, Hargreaves IP. Determination of
389 coenzyme Q10 status in blood mononuclear cells, skeletal muscle, and plasma by HPLC with di-
390 propoxy-coenzyme Q10 as an internal standard. Clin Chem 2005;51:2380-2.
391 [23] Buss T, Aurich J, Aurich C. 2019. Evaluation of a portable device for assessment of motility
392 in stallion semen. Reprod. Dom. Anim. 54: 514-519.

393 [24] Hosoe K, Kitano M, Kishida H, Kubo H, Fujii K, Kitahaara M. Study on safety and
394 bioavailability of ubiquinol (Kaneka QH) after single and 4-week multiple oral administration to
395 healthy volunteers. Regul Toxicol Phamacol 2007;47:19–28.

396 [25] Sinatra ST, Chopra RK, Jankowitz S, Horohov DW, Bhagavan HN. Coenzyme Q10 in
397 equine serum: response to supplementation. J Equine Vet Sci 2013;33:71-3.

398 [26] Bhagavan HN, Chopra RK. Plasma coenzyme Q10 response to oral ingestion of coenzyme
399 Q10 formulations. Mitochondrion 2007;7S:S78–88.

400 [27] Bank G, Kagan D, Madhavi D. Coenzyme Q10: clinical update and bioavailability. J
401 Evidence-Based Complementary Alt Med 2011;16:129-37.

402 [28] Turunen M, Olsson J, Dallner G. Metabolism and function of coenzyme Q. Biochim
403 Biophys Acta - Biomembranes 2004;1660:171-99.

404 [29] Talevi R, Barbato V, Fiorentino I, Braun S, Longobardi S, Gualtieri R. Protective effects of
405 in vitro treatment with zinc, D-aspartate and coenzyme q10 on human sperm motility, lipid
406 peroxidation and DNA fragmentation. Repro Biol Endocrin 2013;11-81.
407 https://doi.org/10.1186/1477-7827-11-81.

408 [30] Gualtieri R, Barbato V, Fiorentino I, Braun S, Rizos D, Longobardi S, et al. Treatment with
409 zinc, D-aspartate, and coenzyme Q10 protects bull sperm against damage and improves their
410 ability to support embryo development. Theriogenology 2014;82:592-8.

411 [31] Yousefian I, Zare-Shahneh A, Zhandi M. The effect of coenzyme Q10 and α-tocopherol in
412 skim milk-based extender for preservation of caspian stallion semen in cool condition. J Equine
413 Vet Sci 2014;34:949-54.

[32] Nogueira BG, Sampaio BFB, Souza MIL, Costa e Silva EV, Zúccari CESN. Coenzyme Q10 and α-tocopherol prevent the lipid peroxidation of cooled equine semen. Repro Dom Anim 2015;50:1003-10.

[33] Aurich C. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. Anim Repro Sci 2005;89:65-75.

[34] Contri A, De Amicis I, Massimo A, Faustini M, Gramenzi A, Robbe D, et al. Effect of dietary antioxidant supplementation on fresh semen quality in stallion. Theriogenology 2011;75:1319-26.

[35] Nadjarzadeh A, Shidfar F, Amirjannati N, Vafa MR, Motevalian SA, Gohari MR, et al. Effect of Coenzyme Q10 supplementation on antioxidant enzymes activity and oxidative stress of seminal plasma: a double-blind randomised clinical trial. Andrologia 2014;46:177-83.

[36] Varner DD, Johnson L. From a sperm's eye view — Revisiting our perception of this intriguing cell. Proc 53rd Ann Conv Amer Assoc Equine Practnrs 2007. p. 104-77.

[37] Amann RP, Johnson L, Thompson DL Jr, Pickett BW. Daily spermatozoal production, epididymal spermatozoal reserves and transit time of spermatozoa through the epididymis of the rhesus monkey. Biol Reprod 1976;15:586-92.

[38] Janett F, Thun R, Niederer K, Burger D, Hässig M. Seasonal changes in semen quality and freezability in the Warmblood stallion. Theriogenology 2003;60:453–61.

[39] Aurich C. Seasonal influences on cooled-shipped and frozen-thawed stallion semen. J Equine Vet Sci 2016;43:1-5.

[40] Wrench N, Pinto CRF, Klinefelter GR, Dix DJ, Flowers WL, Farin CE. Effect of season on fresh and cryopreserved stallion semen. Anim Repro Sci 2010;119:219-27.

[41] Aurich J, Kuhl J, Tichy A, Aurich C. Efficiency of semen cryopreservation in stallions. Animals 2020; 10:1033; https://doi.org/10.3390/ani10061033

[42] Loomis PR, Graham JK. Commercial semen freezing: Individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim Repro Sci 2008;105:119-28.

[43] Alvarenga MA, Papa FO, Landim-Alvarenga FC, Medeiros ASL. Amides as cryoprotectants for freezing stallion semen: a review. Anim Repro Sci 2005;89:105-13.

Figure Captions

Figure 1. Plasma CoQ10 concentrations during a four weeks control period (C; n = 4), immediately before (B), after days 7, 14, 21 and 28 of daily feeding with 1g CoQ10-ubiquinol, and following its cessation (days 35, 42, 49 and 56). * signifies values that were significantly greater ($p < .05$) than at B or during C.

Figure 2. Percent changes in total motility of semen in 3 stallions receiving 1g ubiquinol daily in feed for 4 weeks compared to 4 stallions eating the same diet except for the ubiquinol, following cooling for 24 hr (A), post-thawing after cryopreservation following collection of the ejaculate (B), and post-thawing following cryopreservation after cooling for 24 hr (C). Data from individual stallions were normalized to better reflect changes from day 0 (100%) values. * denotes changes associated with feeding ubiquinol that were different to those under control conditions ($p < 0.05$).

Figure 3. Percent changes in progressive motility of semen in 3 stallions receiving 1g ubiquinol daily in feed for 4 weeks compared to 4 stallions receiving the same diet except for the ubiquinol, following cooling for 24 hr (A), post-thawing after cryopreservation following collection of the ejaculate (B), and post-thawing following cryopreservation after cooling for 24 hr (C). Data from individual stallions were normalized to better reflect changes from day 0 (100%) values. * denotes changes associated with feeding ubiquinol that were different to those under control conditions ($p < 0.05$).

Figure 4. Percent changes in viability (%) of semen over a 4 week period from 3 stallions receiving 1g ubiquinol daily in feed compared to 4 stallions eating the same diet except for the ubiquinol, following cooling for 24 h (A), post-thawing after cryopreservation following

collection of the ejaculate (B), and post-thawing following cryopreservation after cooling for 24 hr (C). Data from individual stallions were normalized to better reflect changes from day 0 (100%) values. * denotes changes associated with feeding ubiquinol that were different to those under control conditions ($p < 0.05$).

Figure 5. Total motility, progressive motility and viability of spermatozoa after 24 h cooling (T_1), before (day 0), during (days 14 and 28), and after (days 42 and 56) four weeks of daily feeding of 1g CoQ10-ubiquinol. * indicates values on day 0 that were lower than those on subsequent days.

Figure 6. Total motility, progressive motility and viability of spermatozoa after cryopreservation soon after collection (T_2) before (day 0), during (days 14 and 28), and after (days 42 and 56) four weeks of daily feeding of 1g CoQ10-ubiquinol. * indicates values on day 0 that were lower than those on subsequent days.

Figure 7. Total motility, progressive motility and viability of spermatozoa after cryopreservation following 24 h of cooling (T_3) before (day 0), during (days 14 and 28), and after (days 42 and 56) 4 weeks of daily feeding of 1g CoQ10-ubiquinol. * indicates values on day 0 that were lower than those on subsequent days.