Epigallo-catechin-3-gallate (EGCG) and green tea polyphenols do not improve stallion semen parameters during cooling at 4°C.

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Abstract

Stallion semen storage for artificial insemination is mainly based on liquid cooled storage. In many stallions this technique maintains sperm quality for an extended period of time (24-72 h) at 7° C.

While this technique is commonly used in the horse industry, there can be a decline in fertility in some stallions, due to an inability of their sperm to tolerate the cool-storage process. The aim of the present work was to evaluate the effect of two natural antioxidants (Epigallo-catechin-3gallate EGCG at 20, 60, 120 μM and green tea polyphenols, P, at 0.001, 0.01 and 0.1 mg/mL) on some sperm parameters (sperm motility, viability/acrosome integrity, and DNA quality) in extended semen immediately after its collection (T0) and after 2, 6, 24 and 48 h of cool-storage.

Two ejaculates from three trotter stallions were analyzed after 48 h of storage at 4° C.

No beneficial effect on the analyzed parameters was observed: the two antioxidants were not able to improve sperm quality after 48 h of storage.

These results are in agreement with previous findings on the effect of different antioxidants reported by other researches, who have demonstrated that stallion semen keep good antioxidant capacity after dilution for 24 h.

In conclusion, the positive effect exerted by antioxidant molecules in other species is not confirmed in the equine one.

Introduction

Equine industry uses assisted reproduction techniques for breeding. One of these techniques, cool-shipped semen, reduces sperm metabolism through cooling and allows a short-term preservation of stallion semen so that it can be shipped while it is still maintaining a good quality (Aurich., 2008; Rigby et al., 2001).

Fertility in some stallions is dramatically reduced following the shipping process. The sperm quality of cool-shipped semen may be affected by semen collection technique, chemical composition of the extenders, centrifugation for removal/reduction of seminal plasma, cooling rate and storage (Rigby et al., 2001; Barbas et al., 2009; Cuervo-Arango et al., 2014; Aurich et al., 2007). Cooling semen exposes spermatozoa to stresses, generally known as "cold shock", resulting in a loss of cell viability, motility and, at the end, fertilizing ability (White, 1993). In addition, stallion sperm may be more susceptible than that from other species to cold shock due to the low cholesterol content of its plasma membrane (Darin-Bennett and White, 1977). These negative effects have been recognized to be caused also by hyper-production of reactive oxygen species (ROS), whose main target are membrane phospholipids and DNA (Ball et al., 2001a; García at al., 2011; Aurich et al., 1997). Spermatozoa are particularly sensitive to ROS, partially because, when deprived of seminal plasma, they lose most of their antioxidant apparatus; secondarily, stallion spermatozoa are highly dependent on oxidative phosphorylation for their metabolism (Bucci et al., 2011; Ball et al., 2001b) and, as a consequence, their mitochondria produce a high amount of ROS.

To date, several antioxidants have been tested to preserve stallion semen from oxidative stress with different and conflicting results. Resveratrol (Giaretta et al., 2013) did not give any result at low doses and exhibited negative effects at high doses, ascorbic acid (Aurich et al., 1997) showed a positive effect on sperm membrane integrity, while catalase did not exert any positive effect. Pyruvate exerted positive effects on sperm motility, while xanthurenic acid did not (Bruemmer et a., 2002); melatonin showed positive results on apoptosis like changes and lipid peroxidation (da Silva et al., 2011), and the addition of superoxide dismutase to the cooling extender was beneficial as well (Cocchia et al., 2011). Finally, quercetin exerted positive effects on stallion semen after freezing (Gibb et al., 2013).

Green tea extracts contain the polyphenolic compound epigallocatechin-3-gallate (EGCG), a powerful antioxidant which removes free radicals by reacting with hydrogen, alkoxyl or peroxyl radicals and chelating iron (Plaza Davila et al., 2015). In addition, EGCG indirectly increases the antioxidant potential by removing free radicals and by stimulating catalase activity (Schroeder et al. 2008).

Recently, green tea phytocomplexes or polyphenols have been used in dog cooled semen (Wittayarat et al., 2012, 2013); an increase of spermatozoa lifespan and motility was observed.

The aim of the present study was to test the effect of different doses of EGCG and green tea extract polyphenols (P) on stallion sperm quality during storage at 4°C for 48 h.

Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Milan, Italy).

Experimental design

Semen collection and preparation

Two ejaculates were collected from three trotter stallions of proven fertility, individually housed at the National Institute of Artificial Insemination, University of Bologna, Italy, using a Missouri artificial vagina with an inner liner and- inline filter to separate the gel fraction (Nasco, Fort Atkinson, WI, USA). Ejaculates were immediately evaluated for volume (in a 50 mL Falcon tube) and concentration (NucleoCounter SP 100, Chemometec, Denmark). Semen was diluted to a final concentration of 30 x 10^6 spermatozoa/mL in Kenney's extender and divided into 6 aliquots: CTR (control); E20, E 60 and E120 (EGCG at concentrations of 20, 60, 120 µM, respectively); PLow, PMed and PHigh (Polyphenols at a concentration of 0.001, 0.01 and 0.1 mg/mL respectively).

Sperm motility, viability with acrosome integrity and DNA integrity were assessed at 0, 2, 6 and 24 h of storage at +4°C.

Motility evaluation

Motility was measured using a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12, standard equine setting). Semen was extended to $30x10^6$ sperm/mL, and 1000 cells were analyzed using a fixed-height Makler Chamber. Sperm motility endpoints assessed were: total motility percentage (TM), progressive motility percentage (PM), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), straightness (STR), linearity (LIN) and beat cross frequency (BCF). The settings parameters of the program were the followings: frames per second 60, number of frames 45, minimum contrast 70, minimum cell size 4 pixel, cell size 6 pixel, cell intensity 106; threshold path velocity, 50 mm/s; threshold straightness, 70%; and path velocity cut off, 20 mm/s.

Viability and acrosome integrity

Sperm acrosome intactness assay (fluorescein-labeled Pisum sativum agglutinin [FITC-PSA]/propidium iodide [PI]) was used for evaluating viability and acrosome integrity; cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson, Milan, Italy) with a 488 nm argon-ion laser. Emission measurements were made using 530/30 band pass (green/FL-1) and 585/42 band pass (red/FL-2) filters. Debris was gated out using a forward scatter/side scatter dot plot, and a minimum of 5000 cells per sample was analyzed. Data were acquired using the CellQuest Pro software (Becton Dickinson). Viable acrosome-intact spermatozoa (VAI) were those cells that did not acquire PI and FITC-PSA, whereas nonviable sperm were those with fluorescence red because of PI uptake. Fifty microliters from each treatment sample were diluted with 133 µL of Dulbecco's PBS solution (Invitrogen Gibco, Carlsbad, CA, USA) and stained with 2 µL of PI (Invitrogen Molecular Probes, Eugene, OR, USA; 2.4 mM working solution) and 0.05 mg/mL Pisum sativum. Samples were incubated at room temperature in the dark for 10 min, and then 20 µL of the stained sample was mixed with 400 µL of Dulbecco's PBS solution and subjected to analysis. A flow rate of approximately 300 events/s was used, and a total of 5000 events per sample was evaluated. List-mode data were analyzed by WinList software (Verity Software House, Topsham, ME, USA).

Sperm chromatin structure assay

Sample preparation and processing, as well as flow cytometer adjustments, were performed as previously described (Evernon et al., 2002; Love, 2005). Briefly, an aliquot of each semen sample was immediately frozen and stored at -20 °C until analysis (maximum 2 weeks). The sperm samples were handled individually and were thawed in a 37 °C water bath. Immediately after thawing (30–60 sec), 2 to 7 µL aliquots of semen were diluted to 200 µL in a buffer solution (0.186-g disodium EDTA, 0.790-g Tris-HCI, 4.380-g NaCI in 500-mL deionized water, pH 7.4). This was mixed with 400 µL of acid detergent solution (2.19-g NaCl, 1.0 mL of 2N HCI solution, 0.25-mL Triton X, deionized water quantum sufficit to a final volume of 250 mL). After 30 sec, 1.2 mL of the acridine orange solution were added (3.8869 g citric acid monohydrate, 8.9428 g Na2HPO4, 4.3850 g NaOH, 0.1700 g disodium EDTA, 4 mg/mL acridine orange stock solution (1 mg/mL), water quantum sufficit to 500-mL, pH 6.0). The sample was covered with aluminum foil, placed in the flow cytometer and allowed to pass through the tubing for 30 sec before counting of the cells. The cell flow rate was placed on the low setting for the machine which, based on sperm concentration in the solution, resulted in an actual flow rate of 100 to 200 cells/s. A total of 5000 events was evaluated for each sample. Sperm from a single control stallion were used as a biologic control to standardize instrument settings between days of use. The flow cytometer was adjusted such that the mean green fluorescence was set at 500 channels (Fl-l at 500) and mean red fluorescence at 150 channels (Fl-3 at 150). Data were acquired in a list mode, and analysis was performed using WinList software (Verity Software House). The percent of sperm with abnormal DNA was defined by the parameter DNA fragmentation index (DFI).

Statistical analysis

Data were analyzed by R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). After checking for normal distribution, data were analyzed by an ANOVA test followed by the Tukey post hoc test for multiple comparison. Data are expressed as mean ± standard deviation.

In all statistical analyses, the minimal level of significance was set at $p<0.05$ unless otherwise specified.

Results

Some parameters showed a decrease due to storage time: TM, PM, BCF for motility (see Tables 1, 2, 7) and DNA damage (Table 11). The other parameters, regarding motion characteristics (VCL, VAP, VSL, ALH, STR, LIN) and the percentage of live spermatozoa with intact acrosomes were not negatively influenced by storage time. See Tables 3, 4, 5, 6, 8, 9, 10.

Discussion

It is generally assumed that equine spermatozoa survive quite well at 4° during the first 24 h of storage; then, for some stallions, viability dramatically decreases. Together with the viability drop, also other parameters (such as sperm motility, DNA fragmentation, mitochondrial activity) undergo a loss, as described by Ball et al. (2001a).

Many Authors reported that oxidative stress is one of the main problems stallion spermatozoa undergo during storage (Ball et al., 2001b; Garcìa et al., 2011; Aurich et al., 1997) and that the relative low cholesterol content in stallion spermatozoa membrane (Darin-Bennett and White, 1977) could exert negative effects on the storage outcome.

Different antioxidants have been tested on chilled stallion spermatozoa to minimize the adverse effects of oxidative stress and to improve sperm quality, but the results obtained are sometimes limited or unsatisfactory. In the present study we tested different doses of EGCG and green tea extract polyphenols (plant molecules recognized to exert beneficial effects on spermatozoa; Plaza Davila et al., 2015, Wittayarat et al., 2012, 2013) on stallion sperm stored at 4° C for 48 h.

Our results demonstrated a consistent decrease in some spermatozoa parameters: total and progressive motility, beat cross frequency and DNA fragmentation undergo significant changes during the storage period, usually more evident at 24 and 48 h. On the other hand, our results clearly show that EGCG and the green tea extracts are not effective in reducing the cooling/storage damage: in fact, we did not register any improvement of any of the parameters we analyzed and the decline in sperm quality did not seem to be influenced by the addition of these substances.

One of the most plausible explanation for these results is that we did not eliminate seminal plasma from sperm suspension: we only added a defined medium with or without antioxidants to reach the fixed concentration. It is likely that seminal plasma contents (catalase, sodium-peroxide dismutase, vitamin C, selenium, zinc) continues playing a protective role during sperm storage and that the addition of exogenous molecules does not further improve sperm quality after storage (Ball et al., 2001b; Garcìa et al., 2011 Aurich et al., 1997; Bucci et al., 2011; Ball et al., 2001 a; Giaretta et al., 2013).

In a preliminary experiment, higher concentrations of the two antioxidants have been tested; in particular, green tea extract was used at 0.5, 0.75 and 1.0 mg/mL, as reported in dog by Wittayarat et al. (2012, 2013). These concentrations demonstrated to be toxic for stallion sperm, as 6 h after resuspension a significant decline in sperm motility and viability was observed, with a 100% dead immotile spermatozoa after 24 h (data not shown). We therefore reduced the polyphenol supplementation the toxic concentration. Other authors (Wittayarat et al. 2012, 2013) used the polyphenol extract for a very long storage period (4 weeks at 5° C); these Authors did not find any difference in viability and acrosome integrity in the first study (Wittayarat et al. 2012) while observing , a positive effect on viability in the second one (Wittayarat et al., 2013). The results from these two studies are in agreement with those from the present one, as we did not notice any difference in viability and acrosome integrity parameters during 48 h storage at 4°C. As for motility, we did not notice any effect of either P or EGCG and we registered a decrease in total and progressive motility. At this regard it should be pointed out that Wittayarat et al. (2012, 2013) assessed motility in a subjective way and obtained an indirect motility index, while in our study we assessed motility by a CASA system, that is essentially objective. In addition, those Authors examined their samples during a very long period of time, up to four weeks, and registered some differences at the endpoint of their experimental period, while in the earlier time-points they did not register any difference either in viability or in motility..

It has to be stressed that some species-specific features of spermatozoa do not guarantee the same effects of antioxidants across species.

We already demonstrated that resveratrol is ineffective for improving stallion sperm storage (Giaretta et al., 2013), and that it could even become dangerous if supplemented at high concentrations. Other authors obtained similar results in porcine sperm (Martin-Hidalgo et al., 2013), concluding that this antioxidant is not suitable for sperm preservation.

On the basis of the above cited references (Giaretta et al., 2013; Ball et al., 2001b) and of our results, we may infer that, at least for cooled equine semen, supplementation of the storage medium with natural antioxidants is not useful, in that they seem to exert a negative effect when used at too high concentrations. Other Authors (Kankofer et al., 2005) indeed, demonstrated that diluting seminal plasma (in commercial extenders) for stallion sperm storage at 5°C activates the antioxidant activity of some enzymes, naturally present in seminal plasma from this species, and reduces ROS generation at 24 h. Another study (Pagl et al., 2006) showed that the addition of glutathione peroxidase, superoxide dismutase (SOD) and catalase to extended stallion semen does not improve sperm quality after storage. Those Authors concluded that ROS couldn't be a cause of decreased sperm quality in liquid stored stallion spermatozoa. In contrast, the addition of antioxidant molecules, such as SOD and melatonin, were effective in improving the quality of cool-stored stallion sperm (da Silva et al., 2011; Cocchia et al., 2011).

In a recent study (Plaza Davila et al., 2015) we demonstrated that EGCG helps in protecting stallion sperm function from the negative effect induced by rotenone during in vitro induced capacitation. In that study we did not find any difference in sperm quality parameters (acrosome reaction and viability) between treatments, but we did observe an important difference in heterologous binding capacity which was decreased by rotenone but fully restored by EGCG. . These results could indicate that sperm viability, acrosome integrity, motility and DNA integrity are not affected by the antioxidants both in preservation and capacitating media.

Therefore, it is reasonable to hypothesize that oxidative stress in cooled equine semen is low, as reported by Pagl and co-workers (2006), while it could represent an important limiting factor in cryopreservation (Ortega-Ferrusola et al., 2009).

The effect of antioxidants may vary depending on species as well as on the way semen is processed for preservation (cooled vs. frozen-thawed). Therefore, it is possible that an antioxidant could be effective in chilled semen but not in frozen one, or effective in dog sperm but not in stallion sperm.

In conclusion, no positive effect of EGCG or polyphenol extract was registered in cooled stallion semen up to 48 h storage, and therefore the addition of these substances in cooling medium for stallion sperm storage is not useful.

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Table 1. Total motility percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml. Different superscript in column indicate significant difference within time ($p<0.05$).

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	81,71±7,78^{a}	84,86±4.78^{a}	81,57±12.74^{a}	80,67±6.19^{a}	81,60±8.17^{a}	81,50±10.75^{a}	82,14±9.3^{a}
2h	82,71±4.89^{a}	81,14±8.73^{a}	82,29±5.31^{a}	83,33±8.33^{a}	80,83±10.72^{a}	83,83±7.33^{a}	80,86±4.18^{a}
6h	78,43±6.80^{a}	79,71±8.06^{a}	82,71±5.82^{a}	80,17±6.08^{a}	77,83±7.63^{a}	77,83±5.85^{a}	78,43±10.18^{a}
24h	69,14±12.01^{bc}	66,57±8.12^{bc}	67,00±11.50^{bc}	64,67±16.15^{bc}	69,33±12.08^{bc}	67,50±7.84^{bc}	67,29±8.20^{bc}
48h	49,86±13.40^{c}	51,14±15.16^{c}	49,14±11.81^{c}	51,00±9.65^{c}	55,17±13.93^{c}	54,50±14.11^{c}	51,43±9.52^{c}

Table 2. Progressive motility percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml. Different superscript in column indicate significant difference within time ($p<0.05$).

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	42,14±9.28^{a}	43,14±11.64^{a}	41,86±11.51^{a}	41,50±13.41^{a}	41,20±10.38^{a}	41,00±11.59^{a}	40,43±11.15^{a}
2h	35,00±11.49^{b}	34,57±9.27^{b}	36,14±13.92^{b}	35,00±8.99^{b}	35,83±9.28^{b}	40,00±12.46^{b}	32,43±6.97^{b}
6h	26,71±6.55^{c}	28,43±3.46^{c}	29,00±5.45^{c}	27,33±3.08^{c}	29,00±6.48^{c}	28,50±5.96^{c}	26,14±5.11^{c}
24h	20,57±6.19^{d}	20,57±5.32^{d}	20,43±4.86^{d}	19,00±6.36^{d}	20,33±8.76^{d}	20,00±7.40^{d}	20,14±5.76^{d}
48h	14,57±4.93^{d}	15,43±6.35^{d}	14,00±6.61^{d}	13,17±7.25^{d}	19,00±10.12^{d}	17,50±9.77^{d}	14,86±7.56^{d}

Table 3. Curvilinear velocity (VCL) percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	199,96±18,03	200,99±26,68	198,69±25,38	200,65±33,81	205,94±32,12	200,68±27,10	201,11±25,66
2h	221,91±25,73	208,79±17,11	216,66±2,38	217,52±14,66	195,75±46,16	210,07±27,09	214,11±12,55
6h	227,29±23,68	218,13±22,99	218,66±13,83	224,37±16,45	217,87±22,78	214,08±25,71	222,63±19,55
24h	205,29±34,48	207,30±31,90	213,39±28,93	224,13±38,88	213,83±39,87	216,25±42,08	219,07±37,89
48h	195,34±53,53	208,06±26,08	202,91±25,14	211,78±35,71	206,73±31,86	205,10±28,71	203,13±25,24

Table 4. Average path velocity (VAP) percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	112,71±15,57	112,61±17,76	114,00±19,12	112,72±22,69	115,44±25,06	112,05±16,94	111,49±19,28
2h	118,49±12,18	113,40±8,83	117,80±11,55	117,42±10,45	109,77±19,26	114,77±17,02	116,49±8,19
6h	119,01±19,48	114,23±14,54	115,17±12,61	117,17±16,16	115,78±16,21	112,12±15,81	116,77±12,75
24h	99,70±20,52	103,44±15,02	105,57±14,52	111,28±20,39	106,40±19,46	106,72±20,26	109,63±18,25
48h	93,60±25,61	102,20±12,91	97,64±9,55	101,10±18,35	102,43±14,59	98,68±11,04	96,64±11,39

Table 5. Straight line velocity (VSL) comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	79,07±17,46	77,93±16,36	79,77±17,43	77,32±20,92	81,00±22,32	76,60±16,08	76,59±18,83
2h	75,03±12,16	73,56±13,42	74,31±16,07	75,42±16,07	71,97±13,17	74,92±18,61	71,66±12,12
6h	67,76±16,85	67,53±10,87	68,10±13,76	67,80±12,30	69,28±12,90	67,85±11,29	66,49±11,79
24h	55,37±8,95	57,99±4,58	57,76±6,60	58,73±4,10	58,15±6,50	56,85±5,13	58,56±5,33
48h	50,83±8,20	55,96±6,02	51,26±4,45	50,77±5,95	58,10±9,18	53,92±6,88	51,90±6,28

Table 6. Amplitude of lateral head displacement (ALH) comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	7,24±0,82	7,14±0,42	7,06±0,48	6,95±0,66	7,50±0,53	7,15±0,47	7,23±0,48
2h	7,71±1,32	7,39±0,31	7,20±0,45	7,60±0,62	10,43±6,96	7,17±0,28	7,29±0,29
6h	7,76±0,59	7,51±0,48	7,70±0,54	7,88±0,43	7,43±0,39	7,78±0,53	7,54±0,46
24h	8,24±0,65	8,00±0,91	7,97±0,80	8,17±1,28	8,25±1,19	7,88±0,81	8,00±0,89
48h	8,21±1,97	7,84±0,88	7,99±1,04	8,60±1,01	8,18±0,66	8,02±0,65	8,30±0,66

Table 7. Beat cross frequency (BCF) comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml. Different superscript in columns indicate significant difference within time ($p<0.05$).

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	37,56±2,28^{ab}	37,14±3,09^{ab}	38,33±2,63^{ab}	38,25±2,22^{ab}	37,10±4,68^{ab}	37,98±3,44^{ab}	36,40±3,41^{ab}
2h	38,29±2,86^{a}	38,83±1,54^{a}	39,47±1,75^{a}	39,07±2,47^{a}	32,50±14,57^{a}	38,95±3,09^{a}	39,23±2,13^{a}
6h	38,34±3,85^{a}	38,79±3,10^{a}	39,00±2,14^{a}	38,82±3,83^{a}	38,68±3,45^{a}	38,55±3,45^{a}	39,01±2,25^{a}
24h	35,24±3,57^{bc}	35,69±2,29^{bc}	35,71±2,53^{bc}	35,75±3,06^{bc}	35,55±2,83^{bc}	36,72±2,88^{bc}	36,91±2,45^{bc}
48h	33,26±5,46^{c}	34,71±3,65^{c}	33,64±3,67^{c}	33,42±3,76^{c}	36,12±4,38^{c}	33,50±2,47^{c}	33,30±2,24^{c}

Table 8. Straightness (STR) percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	69,71±5,79	69,00±7,59	69,43±7,07	69,00±7,72	69,20±6,30	68,33±7,26	68,29±7,23
2h	64,00±8,64	64,86±8,25	63,86±9,74	63,83±8,06	66,00±8,10	65,83±10,17	61,71±6,42
6h	58,29±5,50	59,86±5,64	59,71±5,31	58,67±3,14	60,67±6,12	60,67±6,25	58,14±6,15
24h	56,86±3,93	58,00±5,86	56,57±3,95	55,50±7,09	57,17±8,80	56,00±8,20	56,14±7,17
48h	57,43±11,57	57,14±8,73	55,00±7,70	53,00±10,37	58,00±9,76	57,00±10,64	56,14±9,23

Table 9. Linearity (LIN) **%** comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	42.00±5.13	41.14±5.76	42.29±5.71	41.67±6.25	41.00±5.70	40.50±5.68	40.29±5.96
2h	37.29±6.16	37.29±5.94	37.14±6.91	37.00±6.81	40.67±11.29	38.17±7.05	36.29±4.61
6h	32.29±4.64	33.57±3.41	33.57±5.13	32.50±3.28	34.00±4.47	33.67±3.83	32.57±3.95
24h	28.71±2.36	30.57±3.64	29.29±2.98	28.83±4.58	29.67±4.89	28.67±4.37	29.29±4.61
48h	29.57±8.58	29.43±5.22	27.86±5.15	26.33±6.19	30.17±6.62	28.33±6.59	27.71±5.41

Table 10. Viability and acrosome intactness comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	56.35±10.11	60.67±9.81	58.08±9.75	57.32±9.32	58.12±9.73	58.71±10.75	58.74±7.71
2h	54.18±6.82	56.06±8.41	56.22±7.09	53.63±8.78	55.17±7.77	55.04±9.27	55.13±6.64
6h	54.54±6.19	56.04±7.28	56.27±5.77	55.94±5.53	56.65±5.24	56.45±5.63	56.39±5.98
24h	54.53±7.13	59.02±7.39	58.85±7.51	58.01±7.69	60.25±7.51	60.11±7.14	58.02±7.43
48h	49.72±14.11	56.41±8.26	56.51±6.13	53.91±9.64	59.89±5.53	58.19±5.17	57.65±5.28

Table 11. Chromatin damage % comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml. Different superscript in column indicate significant difference within time ($p<0.05$).

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	24,63±7.24^{a}	30,61±10.01^{a}	30,84±11.37^{a}	31,03±11.52	31,46±11.90	29,74±8.60	27,42±7.74
2h	29,53±10.67^{ab}	40,26±25.74^{ab}	39,67±27.26^{ab}	40,73±28.15^{ab}	43,22±24.84^{ab}	40,62±29.90^{ab}	37,76±22.38^{ab}
6h	38,87±23.52^{b}	43,15±27.89^{b}	41,49±22.98^{b}	44,27±28.03^{b}	44,26±27.88^{b}	38,38±24.91^{b}	38,22±22.35^{b}
24h	42,30±25.90^{b}	38,31±20.0^{b}	39,47±14.95^{b}	43,54±24.13^{b}	44,23±21.16^{b}	38,00±14.90^{b}	34,85±13.34^{b}
48h	42,05±20.51^{b}	42,91±16.95^{b}	40,15±14.10^{b}	41,98±15.05^{b}	47,41±15.46^{b}	45,95±22.25^{b}	45,59±19.29^{b}