PW170 - Semen freezability of endangered Old Kladruber horse – a pilot study

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The Old Kladruber horse has been bred in the Czech Republic for more than four hundred years and has been used for ceremonial purposes and aristocracy all over Europe. However, the current population of this breed is closed and small with the undesirable consequence of increased inbreeding which might be connected with impaired fertility. The aim of this study was to evaluate sperm characteristics of Old Kladruber stallions cryopreserved in two packaging systems. The sperm rich fraction of fresh semen was collected from ten Old Kladruber stallions. Each stallion was collected three times and samples were pre-diluted and centrifuged (650 x g/15 min). Afterwards, Gent extender (MiniTube, Germany) with 20% (v/v) of egg yolk was added to obtain a final concentration at least 250 x 106 of progressively motile spermatozoa/ID. Extended semen was loaded either into 0.5 ml plastic straws or into 5 ml aluminum tubes. Horizontal freezing was performed in a styrofoam box (Animal Reproduction systems, Chino CA, USA). Motility was measured using CASA (Nis-Elements, ver. 4.30, Laboratory Imaging, Prague, Czech Republic). The following kinematic parameters of sperm motility were evaluated: curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s) and average path velocity (VAP, μm/s). Viability was evaluated using carboxyfluorescein diacetate and propidium iodide. The incidence of apoptotic sperms was evaluated with Yo-Pro1 staining using propidium iodide and 4',6-diamidino-2-phenylindole (DAPI). Ten insemination doses from each stallion (5x 0.5 ml straws and 5x 5 ml aluminium tubes) were evaluated after thawing. The student's t-test was used for statistical evaluation and differences between packing systems were considered significant for $P < 0.05$. Kinematic motility parameters of sperms in 0.5 ml straws were higher than in 5 ml aluminium tubes ($P < 0.05$). VAP, VCL and VSL values for 0,5 ml straws were 76 ± 37, 137 ± 63 and 67 ± 37 and in 0.5 ml straws, respectively. In aluminium tubes the VAP, VCL, and VSL values were 64 ± 31, 115 ± 63 and 59 ± 37, respectively. The membrane integrity of the sperms was higher in 5 ml tubes (57 ± 15 %) than in 0.5 ml straws (48 ± 13 %; $P < 0.05$). Lower incidence of apoptotic cells was found in 0.5 ml straws (3 ± 1 %) compared to 5 ml tubes (7 ± 3 %; $P < 0.05$). We conclude based on the results of this study that 0.5 ml straws seem to be more suitable for freezing of Old Kladruber stallions semen. This work was supported by grant NAZV QJ1330189.

Keywords: stallion, sperm, cryopreservation, straw, aluminum tube

PW171 - Influence of Mini-Percoll techniques on motility parameters of ram frozen–thawed sperm

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The aim of this study was to compare the influence of different forces and time of centrifugation in mini-Percoll techniques with traditional Percoll for sperm selection on motility parameters of ram frozen-thawed sperm. Commercial semen from 10 rams of Santa Inês breed, aging 2–5 years old, were used. Samples were obtained from a pool of five straws per animal and divided into five treatments. At post-thawing (PT), the motility parameters were evaluated by Computer Assisted Sperm Analysis (CASA) using the Sperm Class Analyzer (SCA®) system. For traditional Percoll, a 2 mL-gradient (90/45% density) was subjected to a 700 x *g* centrifugation for 10 min followed by 200 x *g* for 5 min. The mini-Percoll (MP) techniques consisted in an 800 μL-gradient (90/45% density) subjected to either: I) two centrifugations of 5000 x *g* for 5 min; II) two centrifugations of 2500 x *g* for 5 min; III) two centrifugations of 1250 x *g* for 5 min; IV: 700 x *g* for 10 min, followed by 200 x *g* for 5 min. At the end of all treatments, aliquots (post-protocols = 0 h) were taken for evaluation of sperm recovery and motility. Afterwards, samples of all protocols were submitted to incubation at 37 °C, 1 h, 2 h and 3 h and the motility was assessed. The variables were subjected to either ANOVA or

Kruskal-Wallis tests depending on normality, Tukey and Fisher-LSD analysis (P < 0.05). The sperm recovery rate (%) was similar (P > 0.05) among all treatments (Percoll: 6.5 ± 1.5; MP-I: 10.8 ± 1.7; MP-II: 8.8 ± 1.4; MP-III: 9.3 ± 1.0; MP-IV: 10.1 ± 1.2) as well as the majority of motility parameters assessed. Only curvilinear velocity (VCL; µm/s) was higher (P < 0.05) after the MP-II (64.5 ± 6.0), MP-III (66.2 ± 5.5), and MP-IV (67.4 ± 5.2), when compared to traditional Percoll (46.3 ± 4.9). Regardless of the incubation (average from 0 to 3 h of each treatment), there was no difference (P > 0.05) among all treatments. The rate of total motility (52.3 ± 2.7%), progressive (19.7 ± 1.9%), and fast progressive (20.0 ± 2.8%), was higher (P < 0.05) in PT compared to any incubation interval, regardless of the protocol used (average of all treatments, considering only the moment of evaluation). After 1 h of incubation, the motility and progressive sperm parameters decreased (P < 0.05) dramatically compared to 0 h. For VCL (73.1 ± 3.3 µm/s), straight line velocity (VSL; 53.2 ± 3.6 µm/s), average path velocity (VAP; 62.0 ± 3.6 µm/s) and lateral head displacement (ALH; 2.5 ± 0.1 µm), the PT values were higher (P < 0.05) than any incubation interval, regardless of the protocol used. Similarly, 0 h was higher (P < 0.05) than any incubation interval. At VCL, VSL, VAP, linearity (LIN), straightness (STR) and ALH there was no difference (P > 0.05) between 1 h and 2 h and between 2 h and 3 h, but the values were higher (P < 0.05) at 1 h compared to 3 h, regardless of the protocol used. Wobble (WOB) and beat cross frequency (BCF) values were higher (P < 0.05) at 1 h when compared to both the 2 h and 3 h. For the first time, we have demonstrated that the reduction of the gradient volume and time of centrifugation, and the increase of its force at Percoll, can be successfully used for ram sperm selection. In conclusion, mini-Percoll may be used instead of traditional Percoll, decreasing costs and time of sperm handling, without motility damages in ram frozen–thawed sperm.

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PW172 - Resumption of ATP levels and mitochondrial functionality in vitrified/warmed ovine oocytes.

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Alterations in mitochondrial functionality and ATP production have been documented in cryopreserved oocytes. However the capability of vitrified-warmed oocytes to recover these damages associated to cryopreservation has not been established yet. The aim of this study was to evaluate the resumption of mitochondrial activity in vitrified ovine oocytes during the post-warming culture. Metaphase II (MII) oocytes were vitrified according to standard procedure used in our laboratory. After warming, oocytes were cultured in 5% CO_2 in air at 39°C. At fixed time-points (0, 2, 4 and 6 hours) the oocytes were processed for: 1) determination of intracellular ATP levels by capillary electrophoresis; 2) assessment of active mitochondrial distribution using MitoTracker Red CM-H_2XRos probe and analyzed through confocal microscopy. For each time-points, n=20 vitrified-warmed oocytes plus n=20 fresh MII oocytes (as a control) were used for the analyses above described. Differences in mitochondrial activity and ATP concentration between control and vitrified oocytes were assessed by ANOVA, while differences in mitochondrial distribution patterns were analyzed by Chi square test. ATP levels were higher (P 0.01) in fresh than in vitrified-warmed oocytes. Moreover differences have been found in vitrified oocytes during post-warming culture. Indeed, from 0 to 2h of culture we observed a decline of ATP levels without significant differences, while at 4 and 6h post- warming the ATP concentration increased significantly but did not reach the higher value seen in fresh oocytes (P<0.01). Also mitochondrial activity showed higher value in fresh oocytes compared to vitrified ones (P<0.01). In particular during the culture post-warming, the value was significantly lower at 0 and 6h compared to fresh oocytes and to 4h of culture, while at 2h the value was intermediate between the time point 0 and 4h (P<0.01). Referring to mitochondrial distribution patterns, we classified it in three groups: 1) Pattern A: homogeneous FINE; 2) Pattern B: homogeneous GRANULAR; 3) Pattern C: heterogeneous CLUSTERED. In fresh group a lower