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EQUIPURE™ IMPROVES FERTILITY OF AGED SUB-FERTILE STALLIONS AFTER ARTIFICIAL INSEMINATION WITH COOLED-TRANSPORTED SEMEN

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1. Objective

- Evaluating the effect of pre-storage selection of spermatozoa from ejaculates of aged sub-fertile stallions by colloidal density gradient (EquiPureTM) centrifugation on:
- Quality traits of stored-transported semen
- Fertility of stored-transported semen after standard uterine body insemination of the mares

2. Justifications

2.1. Reproductive senescence

- The recent trend toward delayed parenthood raises major concerns because of the adverse effects of aging on the male reproductive system
- Subfertility is often associated with aging in the stallions, and the attendant effects of age on testicular function
- Stallions aged > 10 yr shows a tendency for reduced sperm production efficiency
- Sperm output declines in stallions aged > 12 yr, with the lowest levels were found in stallions older than 20 yr of age
- The decline in sperm motility and morphological normal sperm is associated with decreasing breeding efficiency of older stallions
- Previous studies showed significant reductions in pregnancy rate per service and per cycle in stallions older than 10 yr of age

2.2. Sperm selection

- The female reproductive tract exerts stringent sperm selection mechanisms
- Discontinuous density gradients (EquiPure™, Nidacon) have been used to select populations of higher quality sperm for equine assisted reproductive techniques
- EquiPure™ uses a colloid of silane-coated silica particles for isopycnic separation of homogenous sub-populations of structurally and functionally competent sperm cells
- No studies were published regarding the influence of EquiPureTM on stallion sperm fertility after the conventional uterine body insemination of the mares

3. On- STUD FARM PROCEDURES

The experiment was carried out as a part of an AI program in 5 private stud farms (April-August; North Greece; 22°58'E Longitude, 40°37'N Latitude, 32 m altitude)

3.1. The Stallion

Four purebred Egyptian Arabian stallions were used. The stallions were considered sub-fertile based on their seasonal pregnancy rates during the last three consecutive breeding seasons (live covers):

Stallion	Age (yr)	Seasonal pregnancy rate (%)
I	18.0	41.8 (23/55)
II	19.2	59.1 (42/71)
III	20.7	37.2 (16/43)
IV	22.0	32.8 (19/58)

Ejaculates were randomly collected by a CSU-model AV and evaluated for volume, subjective sperm progressive motility (SPM) and sperm concentration (a Neubauer hemocytometer chamber). The mean (±SEM) values of gel-free semen traits were:

Stallion	Ejaculate	Volume (ml)	SPM (%)	x 10^9 spz/ml
I	n= 7	24.9±3.1	43.2± 3.3	0.27±0.07
II	n= 8	21.3±3.0	55.1±3.6	0.32±0.08
III	n= 9	34.7±4.8	51.9±3.9	0.21±0.08
IV	n= 8	28.5± 3.4	45.4±3.1	0.26±0.09

Each gel-free ejaculate was diluted 1:1 at 37°C with a ready-to-use semen extender (INRA 96, IMV Technologies) and split into 2 portions:

The first portion untreated semen

- Semen was transferred to 15-ml conical centrifuge tubes and centrifuged at 500xg for 10 min at room temperature (RT)
- The supernatant was aspirated and sperm pellet was suspended in the semen extender at 30°C to a final concentration of 0.05x10^9 sperm/ml
- Processed semen was then pooled in 50-ml plastic tubes and cooled to 15°C over a 1.6-h period (− 0.7°C/min from 30 to 19°C and − 0.05°C/min from 19 to 15°C) in a styrofoam box filled with 600 ml water at 30°C
- Semen was aliquoted into AI doses and stored under aerobic conditions.
- Each AI dose (10 ml processed semen containing 0.5x10^9sperm + 10 ml air) was loaded into a 20-ml plastic plunger syringe and the syringe was stored-transported horizontally in a cooling transport box (Minitüb, Germany) at 15°C for 6 h

The second portion EquiPureTM-treated semen

Step 1: Sperm concentration
- Semen was initially centrifuged as described above, but sperm pellets were suspended in the semen extender (30°C) at 0.5x10^9sperm/ml

Step 2: Sperm selection
- Concentrated semen (1-1.5 ml/gradient) was layered over 4-ml discontinuous gradients of EquiPureTM (2 ml bottom layer and 2 ml top layer/gradient/tube) in 15-ml conical centrifuge tubes and centrifuged at 300 x g for 20 min at RT

Step 3: Sperm wash [a critical step]
- The supernatant was removed by aspiration
- Each sperm pellet was re-suspended in 2 ml of the semen extender at 25oC
- The re-suspended sperm pellets were pooled in 15-ml conical centrifuge tubes (4 pellets/8 ml/tube) and centrifuged at 500 x g for 10 min at RT

Step 4: Sperm storage
- The supernatant was aspirated and sperm pellets were eventually suspended in the semen extender at 25°C to a final concentration of 0.05x10^9sperm/ml
- Processed semen was then cooled (from 25 to 15°C) over a 1.5-h period, dosed and stored-transported as described above

4.1. The Mare

- Eighty-two reproductively normal, non-lactating, cyclic, single-ovulating mares of light-horse breeds (3-9 years old; body condition score > 5 [1-9 scoring system]), were treated with two IM injections of cloprostenol (250 µg/454 Kg BW, Estrumat®, Schering) 14 days apart
- Mares were then examined by ultrasound every other day until a follicle > 30 mm was detected, and then daily until ovulation
- Mares that developed follicles > 35 mm, were treated with an IV dose of hCG (5 IU/ Kg BW, Chorulon®, Intervet) and inseminated once with 10 ml (one syringe) of stored-transported semen
- Semen was deposited into the uterine body using a flexible AI smooth round tip Universal pipette (Minitüb, Germany)
- At the time of AI, aliquots of stored semen were evaluated for subjective SPM and live morphologically normal sperm in stained smears of buffered eosin-nigrosin stain
- After AI, mares were examined daily to determine the day of ovulation. Pregnancy examinations were performed by ultrasound (5-7.5 MHz, Tringa Linear, Esaote Pie Medical, The Netherlands) on day 60 post-ovulation.

5. DATA ANALYSIS & RESULTS

5.1. Sperm Quality

Influence of EquiPure™ treatment and stallion on percentage of progressively motile spermatozoa with intact plasma membrane and normal morphology of cooled-stored semen

Stallion	Untreated semen	EquiPure-treated semen	Overall
I (n=7)	35.0±3.2 a	48.8±3.8 b	42.0±2.7 A
II (n= 8)	22.5±4.2 a	39.7±3.6 b	32.2±3.1 B
III (n= 9)	14.1±3.5 a	31.9±3.1 b	25.6±2.8 B
IV (n= 8)	29.5±3.9 a	43.5±3.2 b	37.9±2.9 AB
Overall	25.4±3.4 c	40.1±3.0 d	31.5±2.6

- n= number of ejaculates used
- Data were normalized by arcsin transformation and analyzed by 2-way ANOVA (2 semen treatments x 4 stallions) and Duncan's multiple range test (SPSS@ Software)
- Means (± SEM) with unlike small letters in the same raw or capital letters in the same column are significantly different
- AB $p < 0.020$
- ab $p < 0.015$
- cd $p < 0.010$

5.2. Sperm Fertility

Influence of EquiPure™ treatment and stallion on per-cycle 60-day pregnancy rate (%) of cooled-stored semen

Stallion	Untreated semen	EquiPure-treated semen	Overall
I (n=7)	37.5 (3/8) a	70.0 (7/10) a	55.6 (10/18) A
II (n= 8)	40.0 (4/10) a	50.0 (6/12) a	45.5 (10/22) A
III (n= 9)	28.6 (2/7) a	44.4 (4/9) a	37.5 (6/16) A
IV (n= 8)	35.7 (5/14) a	66.7 (8/12) a	50.0 (13/26) A
Overall	35.9 (14/39) c	58.1 (25/43) d	47.6 (39/82)

- n= number of ejaculates used
- Data were and analyzed by Chi-squared test (2 semen treatments x 4 stallions) using SPSS® Software
- Values with unlike small letters in the same raw or capital letters in the same column are significantly different
- cd $p < 0.05$ ($X^2 = 4$)

6. CONCLUSION

EquiPure™ improves stallion fertility in AI programs