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Original Research

## Pregnancy in the Caspian Miniature Horse Using Frozen Semen Cryopreserved with the EquiPRO CryoGuard Freeze Medium and Customized Freezing Protocols

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### ABSTRACT

The primary goal of this project was to establish a protocol to freeze the sperm of the Caspian miniature horse in an attempt to start an intensive artificial insemination program to effectively increase the population of this breed, which has been listed as "Critical Rare Breed" by the American Livestock Breed Conservancy and is in danger of extinction. Commercially available equine freezing medium (EquiPRO CyoGuard Complete egg-yolk extender) was used for the initial setup of two different freeze protocols: slow and fast. The fast-freeze protocol had slightly better postthaw results and was used for a fertility demonstration. Five mares of proven fertility, aged 3 to 12 years, were used in the fertility trials, two of which resulted in pregnancy. This is the first report of pregnancy in the Caspian miniature horse using frozen semen, and the results seem to be a promising start to an extensive program to help this endangered breed, although further research on freezing protocols and conditions for this process are necessary to further improve the survival of semen and pregnancy rate.

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## 1. Introduction

Perhaps the most exciting equine discovery of the 20th century was that the Caspian is not a pony, but an ancient breed of miniature horse, previously believed to have been extinct for more than a thousand years. This tiny horse is believed to be a direct ancestor of the oriental horse breeds, and subsequently of all breeds. These animals are extremely rare and were saved from the brink of

extinction in 1965 by Louise Firouze, an American living in Iran [1].

The Caspian miniature horse is a native of the area around the Elborz Mountains and Caspian Sea in Iran. The current theory is that the Caspian is the ancient miniature horse of Mesopotamia. This horse was used by the Mesopotamians in the third millennium BC until the seventh century AD and then was believed to have become extinct [2].

Caspian horses are small horses that carry some of the characteristics of ponies. However, they have certain physical characteristics that do not match those of either ponies or larger horses. Caspian ponies are perhaps the ancestors of the Arab as well as the wild stock from which hot-blooded horses were derived [3].

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The use of frozen semen has been accepted by two of the world's largest breed associations, the American Quarter Horse and American Paint Horse, as a method to produce registered foals. This has stimulated new interest in the potential use of frozen semen technology [4].

Pregnancy rates of mares inseminated with frozen-thawed semen are highly variable (range, 0%-100% per cycle) [5]. These per-cycle pregnancy rates appear to be affected by mare management and other specific factors, such as the number and concentration of progressively motile spermatozoa within an insemination dose, the timing of insemination, and site of semen deposition.

Amann and Pickett (1987) [6] concluded "Perhaps it is unrealistic to assume that spermatozoa from all stallions should be frozen by the same procedure. It may be desirable to optimize extender(s), cooling rate and warming rate for each valuable stallion." Many commercial semen-freezing laboratories have now adopted a split-ejaculate "test-freeze" procedure to evaluate new stallions.

Differences in the ability of sperm to survive cryopreservation exist not only between species (Darin-Bennett and White, 1977) [7] but also among individual male horses within a species. These differences are probably due to inherent differences in sperm biochemistry and metabolism between both species and individuals within a species [8]. Therefore, several different freezing protocols and conditions were tested to determine the best suitable method for the species and conditions used in this trial.

During this procedure, semen from one or more ejaculates was divided and processed using protocols that differ with regard to centrifugation techniques, extender composition, cooling rates, and package type to determine whether the stallion can produce acceptable postthaw quality using one or more of the techniques [6].

## 2. Material and Methods

Ejaculates were collected from four mature stallions of proven fertility using a Missouri-model artificial vagina over one breeding season (2009) that lasted from March to August. These stallions were housed and semen collected at the "Khojir Caspian miniature horse research and Breeding station of Jihad-e-Agriculture."

Immediately after collection, the gelatinous material and extraneous debris were removed by filtering through gauze and aspiration using a 60-mL syringe, and the gel-free portion of the ejaculates were extended 1:1 (semen:extender) in INRA96 (IMV Technologies, France) that had been warmed to 38°C. The extended samples were evaluated for concentration and progressive motility using light and phase contrast microscopy using the preset Stallion settings of the "Sperm Class Analyzer" (SCA) software (Microptics S.L., Spain) before being centrifuged using a temperature-controlled chamber at 38°C (SpermFuge SF 800 Shivani Industries, Mumbai, India) for 10 minutes at 400 g to remove the seminal plasma. Precipitated sperm were resuspended in INRA96 and evaluated again for concentration and progressive motility using the SCA software.

Smears of semen before and after centrifugation, with a density of approximately 200 sperm per slide, were made on clean glass slides. The slides were left to dry at room temperature (approximately 22°C) and stained using the Papanicolaou stain [9], which is suitable for automated sperm morphology analysis [10,11] and can be referred to as one of the standard stains for sperm morphology assessment according to the World Health Organization and the Tygerberg Strict Criteria [12,13].

The stained slides were examined using light microscopy and the SCA software using the 100× oil immersion objective for morphological defects in a standard number of 150 sperm/slide: sperm head abnormalities and acrosome and middle piece defects were detected using the SCA software, and tail defects (bent, coiled, or double bent) were checked and manually added into the software results. The proportions were used by the software to calculate the total morphologically normal and abnormal spermatozoa [14].

The diluted ejaculates (1:1, semen:extender) from the four stallions, which had a minimum of 70% progressively motile sperm, were placed into 50-mL tubes and transferred to the laboratory under the following two conditions (Fig. 1): one sample was transferred at environment temperature (24-28°C), and the second sample was allowed to cool down in a thermal flask for approximately 1 hour (farm to laboratory transfer time under aerobic conditions). The flask contained ice packs covered with a dry cloth, allowing the samples to be gradually cooled to approximately 4°C.

The transfer temperature for each group was standardized up to the time of freezing.

### 2.1. Freezing Procedure

The diluted samples were centrifuged for 10 minutes at 400 g [15]. At least 95% of the supernatant was removed [16-18], and sperm pellets were resuspended in the EquiPRO CryoGuard Complete egg-yolk freezing extender to a final sperm concentration of  $200 \times 10^6$  sperm/mL. The 0.5-mL straws were filled with the extended sperm and frozen using "controlled rate" and "fast freeze" protocols, with subgroups differing in terms of timing, concentration, and thawing configurations to find the optimum freeze condition (Table 1).

A controlled-rate cell freezer (Cryologic, Cryobath freezer control, Australia) was used with two different freezing curves:

1. Controlled-rate protocol 1: straws at room temperature (approximately 20°C) were cooled at 0.5°C/min to 4°C, 10°C/min to -15°C, and 15°C/min to -120°C before being plunged into liquid nitrogen.
2. Controlled-rate protocol 2: straws containing samples already cooled in the thermal flask were cooled at 0.5°C/min from 4°C to 1°C and 10°C/min to -15°C before being plunged into liquid Nitrogen.

Fast freeze was also performed on both of the cooled and room temperature samples using two different time settings:

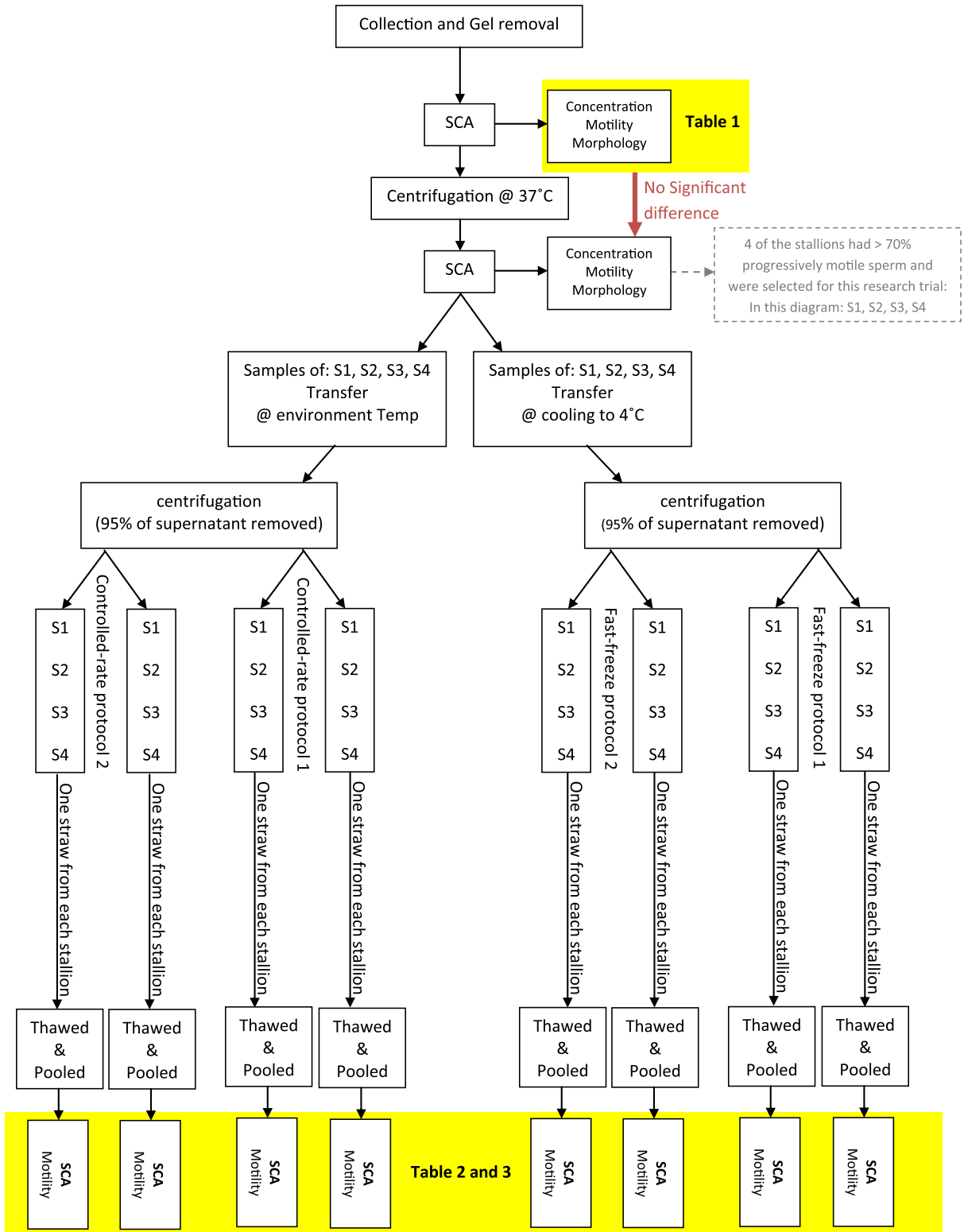


Fig. 1. Flow diagram depicting the study design.

1. Fast-freeze protocol 1: the straws were placed over liquid nitrogen and exposed to nitrogen vapor (3-4 cm above the surface of liquid nitrogen) for 10 minutes before being plunged into liquid nitrogen.

2. Fast-freeze protocol (2): the straws were placed over liquid nitrogen and exposed to nitrogen vapor (3-4 cm above the surface of liquid nitrogen) for 30 minutes before being plunged into liquid nitrogen.

**Table 1**

Postejaculation analysis of sperm using the “SCA” software for concentration, progressive motility, and normal morphology in the final four samples (gel-free semen extended in INRA96) used in the freezing and fertility trial

	Gel-Free Volume (Without INRA96; mL)	Concentration (M/mL)	Total Progressive Motility (%)	Normal Morphology (%)
Stallion 1	21	$85 \times 10^6$	76	44
Stallion 2	25	$71 \times 10^6$	70	53
Stallion 3	29	$83 \times 10^6$	83	49
Stallion 4	24	$91 \times 10^6$	91	47

All frozen samples were kept in liquid nitrogen for at least 4 weeks before being thawed and analyzed or transferred to the mare. The protocol yielding a higher percentage of progressively motile sperm was selected for the subsequent artificial insemination procedures.

## 2.2. Postthaw Evaluation

To perform postthaw evaluation, one straw from each of the four stallions, frozen using the same protocol, was thawed in a 37°C water bath for 30 seconds. The four thawed samples were then pooled before being transferred to the mare to exclude any possible individual characteristic and assimilate transferred sperm as they would be in the field in this study. Before analyzing the percentage of motile and morphological spermatozoa, the pooled samples were diluted 1:1 (v:v) in INRA96 (to a final concentration of  $100 \times 10^6$  sperm/mL).

The percentage of total and progressively motile spermatozoa in each pooled sample was determined in three subdivisions of fast progressive (sperm with fast and linear motility), slow progressive (sperm with nonlinear motility), and nonprogressive (sperm with no progression despite moving tails) at 37°C using a Nikon E200 microscope (Nikon Instruments Inc., NY, U.S.A.) equipped with a heated-stage and phase contrast microscopy using the SCA software (Microptic S.L., Barcelona, Spain). The particulate matter such as yolk droplets were manually identified in the visual result control section of the software and excluded from the analysis. All analyses were performed using the SPSS software program (version 11, SPSS Inc., Chicago, IL).

## 2.3. Artificial Insemination

Five mares at known stages of follicular development and proven fertility, aged 3 to 12 years, were used in the fertility demonstration during the 2009 breeding season. All mares were housed and bred at Khojir Caspian

miniature horse research and breeding station of Jihad-e-Agriculture. All animals were treated humanely; use of these animals followed procedures set forth by the animal ethics committee of Royan Institute, Tehran, Iran.

The mares were not synchronized, and insemination time was based on their natural estrous cycle and daily monitoring. Palpation was performed once every 12 hours from the beginning until the final 48 hours of estrus, followed by constant (every 4 hours) ultrasonographic examinations to determine the time of ovulation before insemination. Two to four hours after the time of ovulation, 3 straws (0.5 mL each) from each stallion (12 straws overall) were thawed and pooled to make the 6-mL insemination dose, which was also analyzed for sperm concentration using the SCA software and adjusted to a concentration of  $\geq 500 \times 10^6$  progressively motile sperm/mL (total sperm count of  $\geq 120 \times 10^7$ ) and a minimum of  $\geq 60\%$  progressive motility. The insemination dose was administered into the uterus using a Universal Insemination Pipette for Equine use (Minitüb, München, Germany). This process was performed only once for each mare.

For pregnancy diagnosis, real-time linear-array B-mode portable ultrasonography machine (Ultra Scan 900, Ami Medical Alliance Inc., Montreal, Canada) fitted with a rectal (linear array) probe was used.

## 3. Results

The diluted gel-free portion of the ejaculates from all stallions was evaluated for concentration, progressive motility, and normal morphology using light and phase contrast microscopy using the SCA software (Microptics S.L., Spain). Only ejaculates from the 4 stallions that had a minimum of 70% progressively motile sperm were selected for freezing trials (Table 1).

The percentage of progressive motility and normal-morphology sperm before and after centrifuging, during the freezing protocol, were not different ( $P > .05$ ).

The percentage of postthaw total motile and progressively motile spermatozoa in each pooled sample revealed that fast freezing of cooled semen exposed to nitrogen vapor for 30 minutes (fast-freeze protocol 2) yielded a higher percentage of progressively motile sperm than all other freezing trial groups (Tables 2 and 3). Therefore, this was selected for the freezing of subsequent ejaculates and artificial insemination.

Early ultrasonographic examinations at 16 days after insemination showed two of five mares to be pregnant, and later examinations at days 40 and 90 after insemination confirmed continuing pregnancies. This is the first worldwide reported pregnancy in the Caspian miniature horse using frozen semen.

**Table 2**

Postthaw analysis of sperm samples used in the fertility trial for total motility, progressive motility, and morphology using the “SCA” software

	Postthaw Total Motile Sperm (%)	Progression Percentage of Total Motile Sperm			Normal Morphology (%)
		Nonprogressive	Slow Progressive	Fast Progressive	
Controlled-rate protocol 1	51	20	21	59	36
Controlled-rate protocol 2	46	21	23	56	34
Fast-freeze protocol 1	59	14	23	63	43
Fast-freeze protocol 2	67	12	20	68	41

**Table 3**

Post thaw analysis of samples used in the fertility trial based on sperm motility types, using the "SCA" software

	Percentage of Total Sperm	Percentage of Motile Sperm
Controlled-rate protocol 1		
Rapid progressive (type a)	30.09	59
Medium progressive (type b)	10.71	21
Non progressive (type c)	10.20	20
Immotile (type d)	49.0	
Controlled-rate protocol 2		
Rapid progressive (type a)	25.76	56
Medium progressive (type b)	10.58	23
Non progressive (type c)	09.66	21
Immotile (type d)	54.0	
Fast-freeze protocol 1		
Rapid progressive (type a)	37.17	63
Medium progressive (type b)	13.57	23
Non progressive (type c)	08.26	14
Immotile (type d)	41.00	
Fast-freeze protocol 2		
Rapid progressive (type a)	45.56	68
Medium progressive (type b)	13.40	20
Non progressive (type c)	08.04	12
Immotile (type d)	33.0	

#### 4. Discussion

The protocol used to transfer semen from the farm to the laboratory in this study was very similar to and supported previous studies [19] in which stallion spermatozoa was centrifuged to remove the seminal plasma at 25°C. The spermatozoa can subsequently be resuspended to a concentration of  $250 \times 10^6$  spermatozoa/mL, cooled to 4°C, and then held at 4°C for 12 hours before freezing without affecting the percentages of motile spermatozoa in the samples after freezing and thawing. This procedure would permit the semen from at least some stallions to be collected, centrifuged on the farm, and shipped to a local facility specializing in equine semen cryopreservation [19].

Pregnancy rates of mares bred using frozen–thawed semen are highly variable (range, 0%–100% per cycle) [5]. Loomis [4] reported that 876 mares were bred using frozen–thawed semen from 106 stallions; the per-cycle and per-season pregnancy rates were 53.5% and 81.9%, respectively.

Similar results were reported by Vidament [20] in a retrospective analysis of 20 years of breeding records within the French National Stud; per-cycle pregnancy rates ranged from 43% to 52%. These aforementioned studies are the results of many inseminations performed by several individuals. Although the number of inseminated mares in this study was quite small, the achieved pregnancy rate (40%) per cycle was similar to the results achieved in previous studies [4,20–22].

In this study, a dose of  $500 \times 10^6$  progressively motile spermatozoa was used based on a previous study by Loomis and Squires [23], which reported a 52.7% per-cycle pregnancy rate when mares were bred using frozen–thawed semen, with doses equal to  $240\text{--}600 \times 10^6$ .

The semen used to inseminate the mares in this study was also similar to the  $500 \times 10^6$  progressively motile spermatozoa dose that has shown to yield maximum pregnancy rates using fresh semen [21,24,25].

In the current study, we successfully used frozen–thawed sperm at the same concentration as recommended for fresh semen [25]. Furthermore, we used a single insemination (2–4 hours after ovulation) rather than the recommended 48-hour interval of inseminations after estrus using fresh semen. Further research to improve insemination success needs to be performed to optimize factors such as insemination time after ovulation, increasing the number/percentage motile sperm, and repeated inseminations shortly after ovulation.

This is the first report of pregnancy in the Caspian miniature horse using frozen semen, and these results seem to be a promising start to an extensive program to assist with the breeding of this endangered breed. This study also suggests that further research on freezing protocols and conditions for this process are necessary to further improve the survival rate of semen and pregnancy in the Caspian miniature horse.

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