Case report

Artificial insemination with frozen semen in rabbits in Brazil: first litter reported

Inseminação artificial com sêmen congelado em coelhos no Brasil: primeiro nascimento reportado

Inseminación artificial con semen congelado en conejos en Brasil: primer nacimiento informado

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ABSTRACT

Brazilian rabbit breeding plays an important social role through family farming. However, one of the challenges faced is the need for new genetic material from genetically improved rabbits. The artificial insemination (AI) technique in Brazil is restricted to the use of fresh or chilled semen. Hence, this is the first report of the use of frozen semen and live kits in Brazil and probably in Latin America. The aims were to evaluate the commercial viability of frozen rabbit semen in Brazil, ranging from cryopreservation to insemination. Three bucks and three rabbit does of Flemish giant rabbits were used. Semen was collected, evaluated and diluted for freezing with CUNIFreeze. Semen was frozen in a 0.5 mL straw, thawed and then filled in a blue curve rabbit AI sheath with the doe in dorsal position with ovulation induced at the same time with buserelin acetate. The ejaculate volume was 0.8 ± 0.8 mL; the mass motility was 3.3 ± 0.6; and the total motility and vigor after dilution were 65% ± 5 points and 3.7 ± 0.6, respectively. Post-thawed, the motility and vigor were 20% ± 10 and 3,0 ± 0,0, respectively. The pregnancy rate was 66.6% with a litter sizes of 5 and 2 kits. Even with a lower result of litter size compared with refrigerated semen, this report brings a new scenario for rabbit breeding in Brazil, showing that frozen semen is a feasible technique for biobanking and mainly bringing the possibility of imported semen from genetically improved bucks solving inbreeding problems.

Key Words: thawed semen; familiar farming; reproductive biotechnologies; cryopreservation

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RESUMO

A criação brasileira de coelhos desempenha um importante papel social através da agricultura familiar. No entanto, um dos desafios enfrentados é a necessidade de novo material genético de coelhos geneticamente melhorados. A técnica de inseminação artificial (IA) no Brasil é restrita ao uso de sêmen fresco ou refrigerado. Portanto, este é o primeiro relato de uso de sêmen congelado e láparos vivos no Brasil e provavelmente na América Latina. Os objetivos foram avaliar a viabilidade comercial do sêmen de coelho congelado no Brasil, desde a criopreservação até a inseminação. Foram utilizados três machos e três fêmeas de coelhos da raça gigante de Flanders. O sêmen foi coletado, avaliado e diluído para congelamento com CUNIFreeze. O sêmen foi congelado em palheta de 0,5 mL, descongelado e, em seguida, aspirado em uma bainha azul de IA em coelhos e inseminado na fêmea em posição dorsal, com a ovulação induzida ao mesmo tempo com acetato de buserelina. O volume ejaculado foi de 0,8 ± 0,8 mL; motilidade massal de 3,3 ± 0,6; a motilidade total e o vigor após a diluição foram respectivamente de 65% ± 5% pontos e 3,7 ± 0,6. Após o descongelamento, a motilidade e o vigor foram respectivamente de 20% ± 10% e 3,0 ± 0,0. A taxa de parição foi de 66,6%, com ninhada de 5 e 2 láparos. Mesmo com um resultado mais baixo da ninhada em comparação com o sêmen refrigerado, este relato de caso traz um novo cenário para a criação de coelhos no Brasil, mostrando que o sêmen congelado é uma técnica viável para biobanco e, principalmente, trazendo a possibilidade de sêmen importado de machos geneticamente melhorados, solucionando o problema de consanguinidade.

Palavras-Chave: sêmen descongelado; agricultura familiar; biotecnologias reprodutivas; criopreservação

RESUMEN

La cría de conejos brasileña juega un papel social importante a través de la agricultura familiar. Sin embargo, uno de los desafíos enfrentados es la necesidad de nuevo material genético de conejos genéticamente mejorados. La técnica de inseminación artificial (IA) en Brasil está restringida al uso de semen fresco o refrigerado. Por lo tanto, este es el primer informe sobre el uso de semen congelado y parásitos vivos en Brasil y probablemente en América Latina. Los objetivos fueron evaluar la viabilidad comercial del semen de conejo congelado en Brasil, desde la criopreservación hasta la inseminación. Se utilizaron tres machos y tres hembras de conejos de raza Flanders giant. El semen se recogió, evaluó y diluyó para congelar con CUNIFreeze. El semen se congeló en una pajita de 0,5 ml, se descongeló y luego se aspiró en una vaina azul AI en conejos y se inseminó en la hembra en posición dorsal, con la ovulación inducida al mismo tiempo con acetato de buserelina. El volumen eyaculado fue de 0,8 ± 0,8 ml; motilidad de masa de 3.3 ± 0.6; La motilidad total y el vigor después de la dilución fueron 65% ± 5% puntos y 3.7 ± 0.6 respectivamente. Después de la descongelación, la motilidad y el vigor fueron 20% ± 10% y 3.0 ± 0.0, respectivamente. La tasa de parto fue del 66.6%, con una camada de 5 y 2 gazapos. Incluso con un resultado de camada más bajo en comparación con el semen refrigerado, este informe de caso presenta un nuevo escenario para la cría de conejos en Brasil, que muestra que el semen congelado es una técnica viable para el biobanco y, principalmente, brinda la posibilidad de semen importado de machos genéticamente mejorados, resolviendo el problema de consanguinidad.

Palabras clave: semen descongelado; Agricultura familiar; biotecnologías reproductivas; criopreservación

Introduction

Produced to cater to the meat, fur, research and pet market, Brazilian rabbit breeding plays a significant role in family farming. The 2017 Census of Agriculture (SIDRA/IBGE, 2017) reports the Brazilian herd at 200,345 rabbits distributed in 16,166 agricultural establishments, of which 77% are directly linked to family farming. The need for improvement of genetic material is among the problems that the activity faces (MACHADO; FERREIRA, 2014), requiring inclusion of new improved genetics from countries having a genetic selection program.

Among the advantages of artificial insemination (AI) in rabbits are improved sanitary control, facilitated genetic trade, reduction in the number of males, and increased organization by grouping animals with similar reproductive stages and batch farrowing (IMV TECHNOLOGIES, 2008). While rabbit semen production centers are well developed in Europe and the technique widely adopted (IMV TECHNOLOGIES, 2008), in Brazil, it was introduced in commercial breeding only in 2016 by Professor Luiz Carlos Machado (IFMG Bambuí) (MACHADO; AMORIM, 2016), growing since then but limited to fresh semen (less than 30 h).

Preserving breeds with a reduced number of individuals tends to be more complicated over time, increasing inbreeding and decreasing genetic variability (DE ROCHAMBEAU; FOURNET-HANOCQ; VU TIEN KHANG, 2000; LOPES; CAROLINO; GAMA, 2004). Some breeds have a broad genetic base in the countries of origin (e.g., Flemish giant in Belgium; German giant in Germany). Frozen semen can contribute to an easier introduction of genetic material from countries with greater heterosis, allowing the maintenance of the Brazilian breeding stock of such breeds, decreasing inbreeding.

Semen cryopreservation is a technique that allows long-term semen storage and transportation and greatly limits the spread of diseases, allowing for more extensive use and continuous stocking of semen from high genetic

merit rabbits, but freezing is problematic in this species due to low sperm membrane permeability (CASTELLINI; BOSCO, 1998). So far, the use of rabbit cryopreserved semen is not a reality on a commercial scale, although it may be useful in breeding programs to maintain genetic diversity (MOCÉ; LAVARA; VICENTE, 2009) and as an option to export genetic material.

The objective of the present study, therefore, was to evaluate the commercial viability of frozen rabbit semen in Brazil, ranging from cryopreservation to insemination.

Material and Methods

This experiment was conducted in a commercial farm located in the city of Vargem Alta (Espírito Santo State, Brazil, 20°40'24.2"S 41°00'30.6"W). The animals were transported (~970 km) one day before to Novagen Genética Ltda located in the city of Itapira (São Paulo State, Brazil, 22°28'47.8"S 46°45'28.6"W) where semen collection followed by semen freezing and insemination were performed. All animals returned to their original location the day after the experiment, where the females proceeded to gestation period and farrowing.

Animals and design: Three bucks and three does of Flemish giant rabbits under reproductive age (~ 1 year old) were used. Males weighed between 6 and 7 kg and multiparous females weighed between 5 and 7 kg and were housed in individual cages, having visual but not physical contact between them. All females were nonpregnant and had no prior exposure to males for the past 60 days. The semen of the 3 bucks was collected and individually frozen. Then, all samples were pooled (heterospermic) to inseminate each doe.

Semen Collection, Processing and Freezing: Semen was collected once per buck in a previously heated glass tube with a complete rabbit artificial vagina (IMV Technologies, Ref. 007220) heated to 42 °C after exposure of males to females. After collection, any gel was removed with a disposable Pasteur pipette. The semen sample was then placed in a water bath at 33 °C, and its initial seminal quality was evaluated by mass motility. Subsequently, the ejaculate was diluted with CUNIFreeze (IMV

Technologies, Ref. 022280) enriched with 20% egg yolk and kept in a water bath at 33 °C at a ratio of 1:6 (v:v, semen:extender) as recommended by the manufacturer. Sperm concentration was not measured. After dilution, a 10 µL drop of diluted semen was placed on a prewarmed slide, covered with a cover slip, and examined using a bright-field microscope (magnification ×200) with a heated stage at 37 °C for motility and vigor evaluation by three technicians. The ensuing result was expressed by consensus. All three ejaculates were frozen regardless of their quality. Inside a cool room, each sample was then placed in a glass beaker with water from the water bath and cooled for no less than 90 min at 4 ºC. Then, the semen was placed in medium bovine semen straws (IMV Technologies, Ref. 005569) and placed on a medium straw freezing rack (IMV Technologies, Ref. 007118). Cryopreservation was performed by placing this rack inside a Styrofoam container filled with liquid nitrogen. Straws were exposed to nitrogen vapor at 5 cm above liquid for 20 min. Afterwards, the straws were immersed in liquid nitrogen and stored in a cryogenic tank until the next day, when AI was performed.

Artificial Insemination: Semen was evaluated for vigor and progressive motility after thawing at 37 °C using a semen thawer (IMV Technologies Brazil, Ref. 450001). Three doses (one of each buck) per doe were thawed and pooled and then filled in a blue curved rabbit AI sheath (IMV Technologies, Ref. 006927). Each doe was then inseminated in the dorsal position (two people, one handler, one inseminator). Just after AI, each doe received 0.42 µg of buserelin acetate (Sincroforte, Ourofino Saúde Animal; im) to induce ovulation. After 12 days, the pregnancy diagnosis was made by ventral palpation by a specialized technician.

Results and Discussion

The results of semen aspects are described in table 1. Two of the three inseminated does were confirmed to be pregnant. Five kits were born in the first litter (3 live and 2 mummified kits) 34 days after AI, and two kits were born in the second litter 35 days after AI, resulting in a 66.6% pregnancy rate and litter size of 3.5 ± 2.1.

Animal	Before Dilution		After CUNIFreeze dilution			Post Thawed	
	Ejaculate Volume	Mass Motility	Motility	Vigor	No. Doses	Motility	Vigor
A	0.30	4	70%	3	4	30%	3
B	0.40	3	65%	4	5	20%	3
C	1.70	3	60%	4	22	> 10%	3

Table 1. Semen parameters before dilution, after extender dilution and after thawing.

To the best or our knowledge and according to the Brazilian Scientific Association of Rabbit Science, the use of frozen semen was never used in Brazil before this trial, allowing us to report for the first time the use of rabbit frozen semen with litter. It is also possible that this is the first report in Latin America.

The ejaculate from each male resulted in semen with sufficient quality for processing, showing good motility and vigor after dilution with CUNIFreeze. Only one male (A) presented semen with a poor acceptable[1] post thawing quality (above 30% motility); however, the three bucks had the doses used at AI. Post-thawing evaluation was difficult due artifacts, possibly lipids from the extender, that saturated the evaluation field and made it difficult to evaluate sperm motility properly.

The litter size obtained (3.5 ± 1.5) was low when compared with results obtained by breeders performing insemination with fresh or refrigerated semen, but the results observed were within those with frozen rabbit semen, where ovulation was induced at the same time as AI was performed (2.8 ± 1.1 to 8.3 ± 0.6) (CORTELL; VIUDES DE CASTRO, 2008; DI IORIO et al., 2018; LIU et al., 2007; MOCÉ; VICENTE; LAVARA, 2003). It is clear that the semen quality results were below expectations. Even if the CUNIFreeze media can be transported at room temperature, it needs to be re-frozen upon receicing. The vial used was stored by us under 4 °C refrigeration only for more than 120 days. This may have caused LDL degradation and pH change, affecting extender quality. Additionally, although the package leaflet recommendation does not require the addition of egg yolk, the addition of the

[1] Although there are no minimum acceptable motility parameters for rabbit semen, such evaluation was performed by co-author PN Jorge-Neto considering his professional experience with cryopreserved semen.

latter as recommended by the technical department of the manufacturer may have yielded artifacts while evaluating the semen. It is clear that the semen extender used for this trial was not kept under the condition recommended by the manufacturer, but even given that, we were able to get pregnancies and litters.

As a strategy for diversification in family farming (GABRIEL et al., 2019), rabbit breeding can play an important social role in Brazil. It is also a suitable activity for sustainable development, being easy to handle, and having high prolificacy and productivity in basically any area. Rabbit meat furthermore has high nutritional quality produced with low water consumption and the use of low nutritional value foods; rabbit production also generates interesting by-products at slaughter and high quality manure (MACHADO; FERREIRA, 2011). One of the current challenges facing the activity in Brazil is the lack of new genetic material availability (MACHADO; FERREIRA, 2014), which could be solved by importing semen from high genetic bucks from countries such as the United States, Spain and France, where the activity is well performed. This obstacle causes a great and fearsome problem for breeders: inbreeding. For this, frozen semen is capable of ensuring safe transport, widespread use and a continuous reserve of genetic material, allowing the diffusion of this new genetic to improve the activity (CASTELLINI; BOSCO, 1998) in Brazil. To date, rabbit AI is limited to the use of chilled semen for up to 30 h in Brazil, restricting inseminator work and transportation between different areas of the country, let alone being able to export Brazilian genetics. Frozen semen will allow this as well as biobanking ancient species and the introduction of new genetic materials solving embryonic disorders and alterations caused by the inbreeding of individuals, even in different regions of the country.

Rabbit semen freezing procedures have been improved, achieving results of born kits similar to fresh semen (fresh 8.7 ± 0.6^{a} vs. 8.3 ± 0.6^{a} frozen) (DI IORIO et al., 2018). Therefore, this work reporting for the first litter with frozen rabbit semen in Brazil allows the market to see a possibility for the future, even though the results of this report were far from ideal due to adverse factors presented in this discussion. It shows the potential to improve the species economical breeding

results and essentially improve the quality of life of Brazilian family farming families. It also shows the need for corrections at the scientific level when researching cryopreserved rabbit semen is carried out, a usual fact for working with something unprecedented at the level of countryside research (not financed by a company).

The natural obstacles caused by the introduction of an unprecedented working technique at the countryside level point to new possibilities for success when working with cryopreservation, culminating in future work about that.

Conclusions

This first report of births with frozen rabbit semen in Brazil brings a new facet for this activity in this country, showing governmental agencies that it is possible to plan for rescue banks and showing the breeders that it is a working technique that can enhance his livelihood through better genetics and planning as long as importing new and better genetic material is possible.

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