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# Effect of calcitriol on the fertilizing capacity of bovine semen cryopreserved *in vitro* and *in vivo*

## Efecto del calcitriol sobre la capacidad fertilizante del semen bovino criopreservado *in vitro* e *in vivo*

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**Abstract.** Previous studies carried out by our laboratory demonstrated that calcitriol capacitates the sperm cells without altering the redox state or sperm viability. The aim of our work was to study the effect of adding calcitriol to the bovine semen extender on cellular metabolism and on the fertilization capacity of cryopreserved spermatozoa *in vitro* and *in vivo*. Semen from one Braford bull was frozen with calcitriol 20 nM (D). Straws without calcitriol were used as controls (C). Spermatozoa cryopreserved with 20 nM calcitriol showed motility (53.3±3.3%), vigor (5.0±0.0) and cell viability (25.4±1.7%) values similar to the C group (46.7±3.3%; 4.3±0.3; 22.9±1.4%). Mitochondrial membrane potential (ΔΨm) was higher in spermatozoa frozen with calcitriol (52.0±0.7% *vs* C: 47.5±0.9%) while ROS levels remained unchanged in the different evaluated groups (C: 140.3±3.5, D: 142.0±8.5 AU). The *in vitro* fertilizing capacity expressed as the percentage of blastocysts developed on the seventh day of culture was 18.03 ± 1.7 % with straws with calcitriol and 15 ± 5.8 % for the control group. When the pregnancy rate was analyzed in D group, the percentage was 11.6 % higher than those inseminated with C straws. In conclusion, the addition of calcitriol to the semen extender of a Braford bull induced a tendency toward increased fertilization capacity of cryopreserved spermatozoa without altering motility, vigor, viability, or ROS levels. Studies with more animals are needed to further investigate the different effects of calcitriol on sperm parameters.

**Keywords.** Calcitriol, semen diluent, sperm parameters, blastocists and pregnancy rates

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**Resumen.** Estudios previos demostraron que el calcitriol capacita a los espermatozoides sin alterar el estado redox ni la viabilidad espermática. El objetivo del trabajo fue estudiar el efecto de la adición de calcitriol al diluyente de semen bovino sobre el metabolismo celular y la capacidad de fertilización de espermatozoides criopreservados *in vitro* e *in vivo*. El semen de un toro Braford se congeló con calcitriol 20 nM (D). Se utilizaron pajuelas sin calcitriol como control (C). Los espermatozoides criopreservados con calcitriol mostraron valores de motilidad (53,3 ± 3,3 %), vigor (5,0 ± 0,0 %) y viabilidad celular (25,4 ± 1,7 %) similares al grupo C (46,7 ± 3,3 %; 4,3 ± 0,3; 22,9 ± 1,4 %). El potencial de membrana mitocondrial (ΔΨm) fue mayor en los espermatozoides congelados con D (52,0 ± 0,7 % *vs*. C: 47,5 ± 0,9 %), mientras que los niveles de ROS se mantuvieron sin cambios en los diferentes grupos evaluados (C: 140,3 ± 3,5; D: 142,0 ± 8,5 UA). La capacidad fecundante fue del 18,03 ± 1,7 % con pajuelas con calcitriol y del 15 ± 5,8 % en el grupo control. El índice de preñez en D fue un 11,6 % superior a C. En conclusión, la adición de calcitriol al diluyente de semen de un toro Braford no alteró la motilidad, el vigor, la utilidad ni los niveles de ROS, pero mejoró el ΔΨm. Estudios con más animales son necesarios para profundizar los efectos del calcitriol sobre los diferentes parámetros espermáticos.

**Palabras clave.** Calcitriol, diluyente de semen, parámetros espermáticos, porcentaje de blastocistos, indices de preñez.

## INTRODUCTION

Utilization of estrus or ovulation synchronization and fixed-timed artificial insemination (TAI) has facilitated the widespread utilization of artificial insemination and can greatly impact the economic viability of cow-calf systems by enhancing weaning weights. Implementation of TAI programs by beef producers results in limited frequency of handling cattle and elimination of the need to detect estrus (Lamb and Mercadante, 2016).

The use of cryopreservation of semen and TAI is a means of genetic improvement stems from the fact that, in most food-producing animals, each ejaculate can be divided into many insemination doses, such us each sire can potentially be used to breed a very large number of females (Parkinson and Morrell, 2019). Although cryopreservation is an exclusive requirement for TAI, its technique produces some negative effects on cells biological parameters, such as modifications in the structure and function of the membrane, metabolic alterations, ionic imbalances, activation of proteases, increased production of reactive oxygen species (ROS) and finally cell death (Ugur et al, 2019). For this reason, new protocols are being developed and cryoprotectant agents are tested for enhanced sperm cryo-survival. Addition of antioxidants like resveratrol (Assunção et al, 2021), curcumin (Gupta et al, 2021), glutathione (Ogata et al, 2022) to bull semen extender resulted in greater sperm quality post-thawing.

Previous studies carried out in our laboratory demonstrated that vitamin D active metabolit, calcitriol (20 nM), induces the capacitation of thawed bull spermatozoa and maintains acceptable values of motility, viability and vigor without altering the redox state and the percentage of cell death (Liaudat et al, 2023). However, the effect of adding calcitriol to the extender and its relationship with reproductive indices has not yet been described. Therefore, the aim of our work was to analyze the effect of adding calcitriol to the bovine semen extender on cellular metabolism and on the fertilization capacity of cryopreserved spermatozoa both *in vitro* and *in vivo*.

## MATERIALS AND METHODS

*Semen collection and cryopreservation*

Sperm cryopreservation was carried out in "El Aljibe" agricultural establishment (General Cabrera, Córdoba, Argentina). An electroejaculator was used to extract the samples and the quality of each ejaculate was analyzed using the ISPERM portable CASA system (Aidmics

Biotechnology, Taipei City, Taiwan). Then, the ejaculate was mixed with the commercial diluent OPTIXCELL (IMV, L'Aigle, France) and a final dilution was carried out to obtain a 0.25 ml straw with $35x10^6$ total sperm each. Calcitriol 20 nM was added to part of the sample and the straws were loaded and sealed. For freezing, an automated freezer model CRYOGEN HSE (Neovet, Uberaba, MG, Brazil) was used and finally the straws were immersed in liquid $N_2$ until their use.

*Experimental design*

To evaluate the effect of adding calcitriol to sperm cryopreservation, 250 µL of bovine semen was thawed and washed once in TALP medium for sperm (Parrish et al, 1988) supplemented with 6 mg/ml of bovine serum albumin (BSA). The following biological tests were then performed: motility, vigor, ΔΨm, viability and ROS levels. Finally, the fertilizing capacity of the sperm with and without calcitriol was evaluated through the percentage of blastocysts and the pregnancy index.

*Sperm motility and vigor*

A drop of each sample was placed between a cover and slide and the percentage of sperm with rectilinear forward movement, with oscillatory movements and completely immobile was analyzed (Liaudat et al, 2023). The vigor of each sample was evaluated according Oliveira et al, 2015.

*Sperm viability*

Viability was evaluated by cell staining with propidium iodide (PI, Sigma, Blois et al, 2021) and was determined in a Guava flow cytometer (Guava easyCyte System, Merck KGaA, Darmstadt, Germany) and then analyzed using FlowJo-V10 software (Tree Star Inc., Ashland, OR, USA).

*Cellular redox state*

Cell suspensions ($2x10^5$) were incubated in the presence of 5-6-carboxy 2,7 dichlorodihydrofluorescein diacetate (carboxy $H_2$DCFDA) 300 µM at 37 ºC for 30 min in the dark. Cells fluorescence intensities were evaluated by flow cytometry (Guava) using the FlowJo program for analysis (Christov et al, 2003).

*Mitochondrial membrane potential (ΔΨm)*

Cell suspensions ($2 \times 10^5$) were incubated in the presence of Rhodamine 123 (10 µg/ml) at 37ºC for 15 min in the dark. The fluorescence intensities of the sperm populations were evaluated by flow cytometry (Guava) using the FlowJo program for analysis.

*In vitro embryo production*

Cumulus cell-oocyte complexes (COCs) were aspirated from ovaries obtained from slaughterhouses. The COCs were selected and cultured in maturation medium for 22 h (Alessio et al, 2016). The semen was thawed and the motile fraction was selected using a Percoll column. Groups of 50 COCs were transferred to plates with fertilization medium (Parrish et al., 1988) and inseminated with $1x10^6$ sperm/ml. After 17 h, the presumed zygotes were removed and transferred to culture drops (SOF medium) where the percentage of blastocysts formed on day 7 of culture was evaluated.

*Heat Synchronization*

The J-Synch (estradiol plus progesterone) protocol was used in females with a body condition score of 3 (scale 1-5). Then, on day 6, the device was removed, 0.15 mg prostaglandin F2α, 300 iu of equine chorionic gonadotrophin (eCG), 5 ml Vit Adapter and 5 ml Adapter min (Laboratorio Biogenesis Bagó) were administered. Subsequently, 70-72 h after removal of the device, 0.10 mg of buserelin was applied and TAI with only one straw was performed in the females that had evidence of heat.

*Pregnancy rate*

After 30 days after the TAI, pregnancy detection was performed by ultrasound, using a MINDRAY 10P device with Doppler (Gurugram, Haryana, India).

*Statistic analysis*

Data were evaluated using ANOVA and Bonferroni test was used as a posteriori.

## RESULTS AND DISCUSSION

**Table 1.** Progressive motility, vigor, ΔΨm and ROS levels of cryopreserved bovine spermatozoa with and without calcitriol

| | Progressive motility (%) | Vigour (1-5) | Viability (%) | ΔΨm (%) | ROS levels (MFI) |
|---|---|---|---|---|---|
| Control | 46.7±3.3 | 4.3±0.3 | 22.9±1.4 | 47.5±0.9 | 140.3±3.5 |
| Calcitriol | 53.3±3.3 | 5.0±0.0 | 25.4±1.7 | 52.0±0.7* | 142.0±8.5 |

*p ≤ 0.05 within column. n:3 per trial for control and calcitriol

Previously published results (Liaudat et al, 2023) showed that treatment of thawed bovine spermatozoa with 20 nM calcitriol for 30 min increases cell capacitation without modifying ROS levels and maintaining acceptable cell viability percentages. It was proposed to add calcitriol to the semen extender in order to evaluate its effect on different biological parameters of the spermatozoa, on the percentage of blastocysts (*in vitro*) and on the pregnancy rate (*in vivo*). The results detailed in Table 1 show that calcitriol increases ΔΨm compared to control cells (spermatozoa frozen without calcitriol). Progressive motility, vigor, viability and ROS levels were not significantly altered by calcitriol, although there is a slight tendency for these parameters to improve compared to control straws. In this sense, Aktar et al (2024) showed that adding 50 ng/ml of vitamin D to the ram egg-yolk extender had a beneficial effect on total and progressive motility, plasma membrane integrity and ΔΨm. Also, extender supplementation with vitamin D 50 ng/mL increased motility and viability in normozoospermic bulls 'sperms did not increase the rate of acrosome integrity compared to the control group (Asadpour et al, 2021).

Subsequently, blastocysts and pregnancy rates of cryopreserved bovine spermatozoa with and without calcitriol were evaluated (Table 2). The results showed that the percentage of blastocysts did not change significantly with calcitriol, although there was a slight tendency to be higher in straws with D. On the other hand, when the pregnancy rate was analyzed, 48.4% (15/31) of the control females were pregnant. These results are in agreement with Bó and Baruselli (2014), who demonstrated that animals treated with progestin-devices with a body condition score >3 could achieve pregnancy rates of 50% or higher. Promising results were observed in females inseminated with straws containing calcitriol, as the pregnancy rate increased by 11.6 % (60%, 6/10) compared to controls.

## CONCLUSION

Our work demonstrates for the first time that calcitriol, active metabolite of vitamin D, increases the blastocysts and pregnancy rates improving the ΔΨm of frozen Bradford sperm and maintains acceptable values of progressive motility, vigor, viability and ROS levels.

**Table 2.** Blastocysts and pregnancy rates of cryopreserved bovine spermatozoa with and without calcitriol. The values represent the means ± SE of each of the treatments.

| | Blastocysts (%) | Pregnancy rate (%) |
|---|---|---|
| Control | 15±5.8 | 48.4 (15/31) |
| Calcitriol | 18±1.7 | 60 (6/10) |

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