PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 43:e07106, 2023
DOI: 10.1590/1678-5150-PVB-7106

Original Article
Livestock Diseases

Viability of *Toxoplasma gondii* in cattle semen cryopreserved with different concentrations of cryoprotectant[1]

Gustavo Felippelli[2]*, João Henrique B. Toscano[2], Weslen F.P. Texeira[3], Isabella B. Santos[2], Breno C. Cruz[2], Willian G. Maciel[2], Vando E. Soares[4], Katia Denise S. Bresciani[5], Welber D.Z. Lopes[6] and Alvimar José Costa[2]

ABSTRACT.- Felippelli G., Toscano J.H.B., Texeira W.F.P, Santos I.B., Cruz B.C., Bresciani K.D.S, Lopes W.D.Z. & Costa A.J. 2023. **Viability of *Toxoplasma gondii* in cattle semen cryopreserved with different concentrations of cryoprotectant.** *Pesquisa Veterinária Brasileira 42:e07106, 2023.* Centro de Pesquisas em Sanidade Animal, Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castellane s/n, Zona Rural, Jaboticabal, SP 14884-900, Brazil. E-mail: gusvetfelippelli@gmail.com

Toxoplasma gondii can be eliminated in bovine semen. Cryopreserved semen is often used due to the fact that artificial insemination in dairy and beef cattle provides benefits in terms of production. However, little is known regarding the viability and infectivity of *T. gondii* tachyzoites in cryopreserved bovine semen. In the present study, cattle semen negative for *T. gondii* were contaminated with 1 x 10^6 tachyzoites (RH strain) and cryopreserved with and without different cryoprotectants, such as DMSO (concentrations of 2.5%, 5.0%, 7.5%, 8.0% and 10.0%) and glycerol (2.25%, 2.5%, 3.0%, 5.0%, 7.5% and 10.0%), followed by freezing in liquid nitrogen (-196°C). After 24 hours, the samples were thawed and inoculated in 10 mice per cryoprotectant concentration. The mice were evaluated for clinical signs of toxoplasmosis (rough coat, diarrhea, hypoactivity and sudden death) as well as serum titers of IgM and IgG and the presence of tachyzoites in the peritoneal lavage. The results revealed that *T. gondii* remained infective in all samples. Clinical signs of toxoplasmosis were observed in the mice beginning with the 6th day post-inoculation (DPI) and 100% lethality was found between the 7th and 9th DPI. Viable tachyzoites were recovered from peritoneal exudate of dead mice (except for the control group), with higher mean of tachyzoite counts in the intraperitoneal lavage for 5% DMSO (±3.32 x 10^6), 8% DMSO (±3.53 x 10^6), 3% glycerol (±4.75 x 10^6), 7.5% glycerol (±6.26 x 10^6) and the absence of cryoprotectant (±3.11 x 10^6). Seroconversion occurred in the treated groups, with titers of IgG from 1:16 to 1:128 and IgM from 1:16 to 1:512. *T. gondii* viability and infectivity were maintained in cattle semen during 24 hours of cryopreservation at -196°C with and without cryoprotectant. However, further studies are necessary to determine whether cryopreserved semen contributes to the spread of toxoplasmosis through artificial insemination.

INDEXING TERMS: *Toxoplasma gondii*, cattle, cryopreservation, cryoprotectant, DMSO, glycerol, toxoplasmosis, semen samples, RH strain, IFI.

[1] Received on June 27, 2022.
Accepted for publication on July 20, 2022.

[2] Centro de Pesquisas em Sanidade Animal (CPPAR), Departamento de Patologia, Reprodução e Saúde Única, Faculdade de Ciências Agrárias e Veterinárias (FCAV), Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castellane s/n, Zona Rural, Jaboticabal, SP 14884-900, Brazil. *Corresponding author: gusvetfelippelli@gmail.com

[3] Graduate Program in Animal Science, Universidade Federal de Goiás (UFG), Goiânia, GO, Brazil.

[4] Universidade Brasil, Descalvado, SP, Brazil.

[5] Faculdade de Medicina Veterinária de Araçatuba (FMVA), Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Rua Clovis Pestana 793, Araçatuba, SP 16050-680, Brazil.

[6] Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás (UFG), Goiânia, GO 74605-050, Brazil.

RESUMO.- [Viabilidade do *Toxoplasma gondii* em sêmen de bovino criopreservado em diferentes concentrações de crioprotetores.] Sabe-se que *Toxoplasma gondii* pode ser eliminado no sêmen bovino. A inseminação artificial em bovinos leiteiros e de corte proporcionou avanços e benefícios nas produções e para isso o sêmen criopreservado é frequentemente utilizado. No entanto, pouco se sabe sobre a viabilidade e infectividade dos taquizoítos de *T. gondii* em sêmen bovino criopreservado. Para isso o sêmen bovino, negativo para *T. gondii*, foi contaminado com $1x10^6$ taquizoítos (cepa RH), criopreservados com ou sem diferentes crioprotetores como DMSO (2.5%, 5.0%, 7.5%, 8.0% e 10.0%) e Glicerol (2.25%, 2.5%, 3.0%, 5.0%, 7.5% e 10.0%) e congelados em nitrogênio líquido (-196°C). Após 24 horas, essas amostras foram descongeladas e inoculadas em 10 camundongos por diluente de concentração de crioprotetor. Os camundongos foram avaliados quanto a sinais clínicos de toxoplasmose (pele áspera, diarreia, hipoatividade e morte súbita), títulos séricos de IgM e IgG e presença de taquizoítos no lavado peritoneal. Os resultados mostraram que *T. gondii* se manteve infectante em todas as amostras, inclusive naquelas sem crioprotetor. Sinais clínicos de toxoplasmose foram observados nos camundongos a partir do 6º dia pós-inoculação (DPI) e 100% de letalidade foi verificada entre o 7º ao 9º DPI. Nos camundongos mortos, exceto no grupo controle, taquizoítos viáveis foram recuperados do exsudato peritoneal, com maior média de taquizoítos quantificados na lavagem intraperitoneal para DMSO a 5% ($\pm3.32x10^6$), 8% ($\pm3.53x10^6$) e glicerol 3% ($\pm4.75x10^6$), 7,5% ($\pm6.26x10^6$) e livre de crioprotetor ($\pm3.11x10^6$). A soroconversão ocorreu nos grupos tratados com títulos de IgG (1:16 a 1:128) e IgM (1:16 a 1:512). A viabilidade e infectividade do *T. gondii* no sêmen bovino durante as 24 horas de criopreservação a -196°C foram mantidas com ou sem crioprotetor. No entanto, mais estudos são necessários para verificar se o sêmen criopreservado contribui para a disseminação da toxoplasmose na inseminação artificial.

TERMOS DE INDEXAÇÃO: *Toxoplasma gondii*, bovinos, criopreservação, crioprotetores, DMSO, glicerol, toxoplasmose, amostras seminais, cepa RH, RIFI.

INTRODUCTION

Toxoplasmosis is a global zoonotic disease caused by *Toxoplasma gondii* (Nicolle & Manceaux 1909), which is an obligatory intracellular parasite with a heteroxenous, facultative life cycle and the ability to infect a variety of animal species, such as sheep, cattle, goats, pigs, horses and poultry (Mello 1910, Quinn & McCraw 1972, Dubey & Beattie 1988, Bresciani et al. 1999, Tenter et al. 2000, Sawadogo et al. 2005, Fialho et al. 2009, Dubey et al. 2013). The main *T. gondii* infection route in cattle is the ingestion of sporulated oocysts present in pastures, silage, feed and water. Moreover, sexual transmission of *T. gondii* (oocysts and tachyzoites) by the seminal route in cattle has been reported for seminal vesicles diagnosed by PCR and bioassays (semen samples) in mice (Scarpelli et al. 2009).

Artificial insemination in beef and dairy cattle using cryopreserved semen is a common management strategy to improve herd genetics and consequently enhance productivity (Boni 2012). Cryopreservation enables the storage of semen for an indefinite period, reducing the risks and costs related to the acquisition and transportation of breeders and facilitating the diffusion of genetic material between distant sites, with the further advantage of reducing the risk of disease transmission (Vishwanath 2003, Castelo et al. 2008, Rodgers et al. 2012, Baruselli et al. 2017).

Cryoprotectants used for sperm preservation include glycerol, ethylene glycol, dimethyl sulfoxide (DMSO), acetamide, lactamide and dimethylacetamide (Fickel et al. 2007, Kashiwazaki et al. 2006). Glycerol is the most common cryoprotective agent used for freezing bovine semen, but can be toxic to sperm cells and cause contraceptive effects (Becker et al. 1977). DMSO at concentrations of 4 and 6% constitutes an alternative to glycerol (Snedeker & Gaunya 1970).

As literature on *T. gondii* cryopreserved in semen is scarce, the aim of the present study was to assess the viability of *T. gondii* tachyzoites in cryopreserved cattle semen with different concentrations of DMSO and glycerol.

MATERIALS AND METHODS

All experimental procedures received approval from the Ethics Committee on Animal Use of the "Faculdade de Ciências Agrárias e Veterinárias" (School of Agrarian and Veterinary Sciences - FCAV) of "Universidade Estadual Paulista 'Júlio de Mesquita Filho'" (Unesp) (certificate number: 026341/13). The experiment was conducted at the Animal Health Research Center and Department of Preventive Veterinary Medicine and Animal Reproduction, Unesp, in the municipality of Jaboticabal/SP, Brazil, and Laboratory of Molecular Diagnosis Applied to Zoonoses of the Department of Veterinary Hygiene and Public Health, "Faculdade de Medicina Veterinária e Zootecnia" (School of Veterinary Medicine and Zootechnics - FMVZ), Unesp, in the municipality of Botucatu/SP, Brazil.

Obtention of *Toxoplasma gondii* tachyzoites. The RH strain (Sabin 1941) was used in the present study. *T. gondii* tachyzoites were maintained by periodic passages in the peritoneal cavity of Swiss mice (*Mus musculus*) approximately one month of age (weight: 20-25g). Tachyzoites were obtained from the peritoneal lavage (400µL of peritoneal exudate; approximately 10^6 tachyzoites) of previously infected mice on the second day after infection (Mineo et al. 1980). The mice were euthanized by cervical dislocation following the guidelines of the National Council for the Control of Animal Experimentation (CONCEA) and positioned dorsally for the injection of 3mL of sterile saline solution into the cavity. Abdominocentesis was then performed for the recovery of *T. gondii* tachyzoites. A drop of the peritoneal lavage was placed on a slide and observed under an optical microscope (objective: 40x) for the subjective analysis of tachyzoite morphology and viability (Costa et al. 2011). Lavages with a smaller number of somatic cells (red blood cells, leukocytes, desquamated epithelial cells) and higher quantity of tachyzoites ($1 x 10^6$) were chosen for semen infection (adapted from Camargo 1964). The selected intraperitoneal lavages were centrifuged twice – once at 1000rpm for 30 minutes for the sedimentation of impurities and then at 2500rpm for eight minutes. The pellet was resuspended in 2.0mL of phosphate-buffered saline (PBS). *T. gondii* tachyzoites ($1 x 10^6$) were submitted to the Trypan Blue exclusion test to assess cell viability and counted in a Neubauer chamber with a volume of 10µL (Mitsuka et al. 1998).

Selection of bulls, andrological examination and assessment of semen characteristics. Blood samples from 23 bulls in reproductive age (≥24 months) from the municipality of Ribeirão Preto in the state of São Paulo, Brazil were collected through jugular venipuncture in vacutainer tubes without anticoagulant. The material was centrifuged at 1000g for 10 minutes and sera

were stored at -20°C until processing. All samples were tested for *T. gondii* (Camargo 1964) and *Neospora caninum* (Conrad et al. 1993, Dubey & Lindsay 1996, Soares et al. 2009) antibodies by indirect antibody immunofluorescence assay (IFA) using a bovine specific anti-IgG conjugate (Sigma Chemical – F7887).

Bulls were considered positive for *T. gondii* when the serum had titers equal to or greater than 1:64 (Camargo 1964) and positive for *N. caninum* with titers equal to or greater than 1:50 (Figliuolo et al. 2004). The animals were also tested for antibodies against *Brucella abortus* using the Card Test (Alton et al. 1988) and antibodies against *Leptospira* spp. (serovar *wolffi, hardjo, patoc, pomona, icterohaemorrhagiae* and *canicola*) using the microscopic agglutination test (Centro Panamericano de Zoonoses 1985).

To determine the presence of *T. gondii* DNA, semen was collected from the 23 bulls through electroejaculation (Mascarenhas & Gomes 1950). DNA was extracted using the Illustra Blood genomicPrep Mini Spin kit (GE Healthcare Life Sciences do Brasil Ltda®, Brazil) according to the manufacturer's instructions, followed by conventional polymerase chain reaction (cPCR) using the primers TOX4 (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3') and TOX5 (5'-CGCTGCAGACACAGTGCATCTGGATT-3'), which amplify a region of 529 bp of 18S rRNA (Homan et al. 2000). DNA from the RH strain of *T. gondii* was used as the positive control. The results were viewed in 1% agarose gel stained with ethidium bromide solution (Fig.1).

Only bulls serologically negative for the diseases evaluated and without *T. gondii* in the semen were selected. One of the 23 bulls was randomly selected. This bull (no. 10) was approximately four years of age, was crossbred (*Bos taurus taurus* x *Bos taurus indicus*), with a good body condition (4 on a scale ranging from 1-5), good overall appearance and no physical abnormalities detected during the clinical examination. The animal was submitted to the andrological examination to determine its reproductive potential following the guidelines of the Brazilian College of Animal Husbandry (CBRA 2013). Examinations were performed of the external genitals (inspection and palpation of the scrotum, testes, epididymis, spermatic cords, foreskin and penis) and internal genitals (vesicular glands, ampulla of the vas deferens, prostate and bulbourethral gland by rectal palpation). A semen sample was collected and analyzed in terms of physical (volume, whirling, vigor, motility and concentration) and morphological characteristics (CBRA 2013).

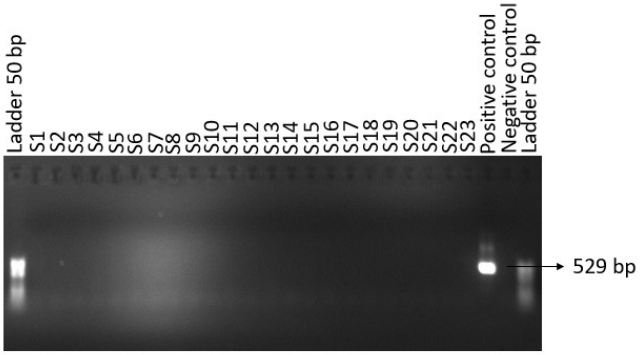


Fig.1. Photography of agarose gel of primary PCR product showing non-bands. Bull semen samples not positive in PCR with primers TOX4 and TOX5. Positive samples should amplify in region of 529 bp (control).

Semen dilution, cryoprotectants and contamination with *T. gondii* tachyzoites. Fifty mL of a single ejaculate were divided into fourteen aliquots of 0.25 mL (containing 20 x 10^6 spermatozoids) and placed in 15-mL Falcon tubes in a water bath (37°C). Twelve of the fourteen semen samples contaminated with *T. gondii* tachyzoites obtained from the peritoneal lavage of experimentally infected mice. A *T. gondii* suspension (0.05 mL, 1 x 10^6 of viable tachyzoites) was added to each aliquot of semen.

After contamination, TRIS (27g/L) + 14g/L citrate (14g/L citric acid + 10g/L fructose) + egg yolk (20% v/v) as diluent were added to each of the samples containing the different concentrations of cryoprotectant (glycerol and DMSO). One mg of streptomycin was also added to each concentration. Five concentrations of DMSO (2.5%, 5.0%, 7.5%, 8.0% and 10%) and six concentrations of glycerol (2.25%, 2.5%, 3.0%, 5.0%, 7.5% and 10%) were used in the experiment based on studies involving mammalian, fish and chicken sperm (Polge et al. 1949, Lovelock & Bishop 1959, Fahy 1986, Fontgibell & Vidal 1998, Agca & Critser 2002, Hubálek 2003, Ribeiro & Godinho 2003, Murgas et al. 2007, Sanches et al. 2008). However, one should bear in mind that, despite citations in the literature, there is no standardization of concentrations.

Two controls groups were established, in which the semen samples did not receive tachyzoites of *T. gondii*, only the diluent (TRIS + citrate + egg yolk) and the cryoprotectants 5% DMSO or 3% glycerol. Another group was established without cryoprotectant and contaminated with *T. gondii* tachyzoites. Two concentrations were first chosen (5% DMSO and 3% glycerol), as lower concentrations of DMSO offer less toxicity on the intracellular level and glycerol at a concentration of 3% is routinely recommended for the cryopreservation of semen at genetic bovine improvement centers.

Freezing of samples using conventional method. The Falcon tubes containing the semen with or without *T. gondii* tachyzoites + diluent + with or without cryoprotectant were then placed in two plastic beakers, which were placed in a refrigerator (4.5°C) for four hours (time required for cooling and equilibration of temperature). The samples were then placed in duly labeled 0.25-mL French straws, which were sealed with modeling clay. Eleven straws were filled per tube of semen. The straws were then distributed horizontally, positioned on an appropriate grid, which was placed on a metal platform with a height of 9cm. The set was placed in a polystyrene box (30cm in height x 19cm in width x 39cm in length) containing a 6-cm layer of liquid nitrogen and the straws were maintained 3cm from the nitrogen vapor (-150°C) for 15 minutes. After this period, the platform was removed and the grid, together with the straws, was immediately immersed in liquid nitrogen (-196°C) to complete the freezing process. With the aid of metal tweezers, the straws were then transferred to the cryogenic cylinder for storage until further analysis (adapted from Duarte & Garcia 1997).

Semen assessment after thawing. For each concentration of DMSO and glycerol, one straw with frozen semen was thawed (water bath at 35°C for 20 seconds) and the semen was immediately submitted to vigor and motility analysis using the methods referenced above.

***T. gondii* feasibility and infectivity in cryopreserved semen samples.** A method adapted from Teale et al. (1982) was used to assess the viability and infectivity of *T. gondii* tachyzoites in cryopreserved (DMSO and glycerol) semen samples. For each of the fourteen semen tubes, 10 straws with semen were thawed (water bath at 35°C for 20 seconds) 24 hours after freezing. Ten mice were inoculated (intraperitoneally) with the total content of one straw (0.25mL) of semen in an equal volume of PBS (Table 1).

The mice were observed daily for 42 days for clinical signs of toxoplasmosis (rough coat, hypoactivity, diarrhea, ascites and eyes half closed), following the method described by Costa et al. (1977). Animals that died by the 10th day after inoculation (acute phase of toxoplasmosis) were submitted to necropsy to search for tachyzoites in the abdominal cavity through an analysis of the peritoneal lavage. Mice that survived until the 42nd day after experimental infection were euthanized for blood collection. Serum samples were submitted to IFA to investigate seroconversion. IFA was performed using anti-mouse IgG and IgM conjugate (Sigma Chemical – F0257 and F9259) produced in sheep. Additionally, brain cysts were investigated in seropositive animals and those that died between the 11th and 42nd day after infection (Camargo 1964).

Statistical analysis. The experimental data were tested for independence, normality and homoscedasticity. Differences in tachyzoite counts recovered from the peritoneal exudates of the bioassay with mice were determined using the Kruskal-Wallis test and mean tachyzoite counts were compared using the Scott-Knott test with a 95% confidence interval ($p \leq 0.05$). Reciprocal serological concentrations (IgG and IgM class antibodies) in the mice were compared using Fisher's exact test with a 95% confidence interval. All analyses were performed using the SAS statistical software, version 9.0.

RESULTS

Cryoprotective activity of DMSO and glycerol in semen samples

Semen samples infected with *Toxoplasma gondii* tachyzoites and with DMSO at concentrations 7.5%, 8.0% and 10% had the greatest vigor (score of 2) and motility (20%). For glycerol, the greatest vigor (score of 4) and motility (35.0%) were found with concentrations of 2.5%, 3.0% and 5.0% (Table 1).

Viability and infectivity of *T. gondii* in cryopreserved semen samples in mice

The following clinical signs were observed in mice: apathy, rough coat and diarrhea from 6th day post-inoculation (DPI) and 100% lethality on average between days 7 and 9. With the exception of the control group (healthy mice seronegative for toxoplasmosis), all mice that died had viable tachyzoites in the peritoneal lavage, with no significant differences among groups ($p>0.05$) determined by Tukey's test (Table 2). However, cluster analysis (Scott-Knott test) revealed a significant difference ($p \leq 0.05$) in the mean number of tachyzoites quantified in the intraperitoneal lavage among the groups experimentally inoculated with *T. gondii* tachyzoites (Fig.2). As the experimental mice that received semen with *T. gondii* tachyzoites died before the 11th DPI, no brain cysts were found.

Scott-Knott cluster analysis demonstrated the highest mean tachyzoite counts recovered from intraperitoneal lavage of experimental mice in the 7.5% glycerol, 3.0% glycerol, 7.5% DMSO and 8.0% DMSO groups ($p \leq 0.05$), along with suitable seminal vigor and motility patterns for bovine reproduction procedures (Fig.2).

Table 1. Spermatic parameters, post thawing, of bovine semen ($20x10^6$ spermatozoids) infected or not with *Toxoplasma gondii* tachyzoites ($1x10^6$) cryopreserved with DMSO and glycerol at different concentrations

Experimental groups	Cryoprotectant agent (concentration)	Semen parameters	
		Vigor	Motility (%)
I	DMSO 2.5%	1	5
II	DMSO 5.0%	1	5
III	DMSO 7.5%	2	20
IV	DMSO 8.0%	2	20
V	DMSO 10.0%	2	20
VI	Glycerol 2.25%	2	15
VII	Glycerol 2.5%	4	35
VIII	Glycerol 3.0%	4	35
IX	Glycerol 5.0%	4	35
X	Glycerol 7.5%	3	25
XI	Glycerol 10.0%	2	5
XII	Crioproctetor free	-	-
XIII	DMSO 5.0% (control)	1	5
	Glycerol 3.0% (control)	4	35

Table 2. Mice mortality, average survival time and *Toxoplasma gondii* tachyzoites (RH strain) quantifications recovered from peritoneal washing after experimental infection with semen containing or not *T. gondii* tachyzoites and crioprotectors (DMSO and glycerol)

Experimental groups	Mortality		Average survival	Tachyzoites x 10^6
	N of deaths/ Total	%	days (PID)	(Rate ± SD)
DMSO 2.5%	10/10 10/10	100 100 8.4	8.4	1.85 ± 0.77[A]
Glycerol 2.25%			1.40 ± 0.29[A]	
DMSO 5.0%	10/10 10/10	100 100 8.4	8.0	3.32 ± 4.55[A]
Glycerol 2.5%			0.55 ± 0.23[A]	
DMSO 7.5%	10/10 10/10	100 100 8.17	7.6	1.76 ± 1.97[A]
Glycerol 3.0%			4.75 ± 5.50[A]	
DMSO 8.0%	10/10 10/10	100 100 8.2	9.2	3.53 ± 5.61[A]
Glycerol 5.0%			1.61 ± 0.82[A]	
DMSO 10.0%	10/10 10/10	100 100 8.2	8.2	1.40 ± 0.19[A]
Glycerol 7.5%			6.26 ± 1.69[A]	
Glycerol 10.0%	10/10	100	8.0	1.40 ± 1.51[A]
Crioprotector free	10/10	100	7.6	3.11 ± 2.46[A]
DMSO 5.0% (control)	0/10 0/10	0 0 42*	42*	0.00 ± 0.00[B]
Glycerol 3.0% (control)			0.00 ± 0.00[B]	

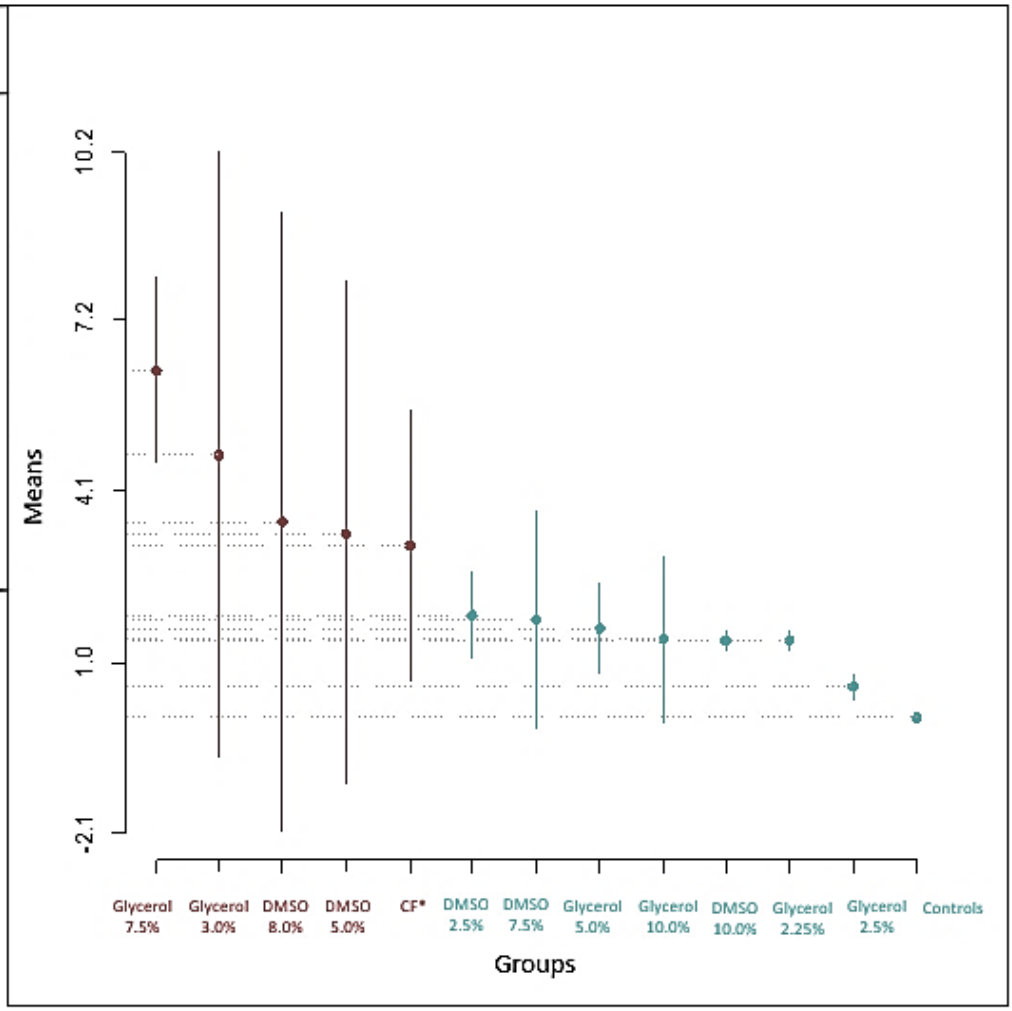
* Control groups: euthanazed animals after the 42nd experimental day; [A,B] Mean values followed by the same letter on the same line do not differ significantly at a Kruskal-Wallis test ($p \geq 0.05$); PID = post-infection day.

IgM and IgG against *T. gondii* in mice

All experimental mice (except the control group) had seroconversion with titers of IgM (1:16 to 1:512) and IgG (1:16 to 1:128). Analyzing the results with DMSO, all five concentrations led to positive mice for the serological IgM titers (acute phase). The titers with the highest number of positive mice were 1:32 and 1:64, with percentages of 36.0% (18/50) and 36.0% (18/50), respectively. Other IgM titers had the following percentages 1:16 (12.0%, 6/50), 1:128 (8.0%, 4/50), 1:256 (4.0%, 2/50) and 1:512 (4.0%, 2/50). Glycerol concentrations (2.25%, 2.5%, 3.0%, 5.0%, 7.5% and 10.0%) had the highest rates of infected mice with titers 1:64 and 1:32 (40%, 24/60 and 31.66%, 19/60, respectively). The lowest rates were found for titers of 1:16 (15%, 9/60), 1:128 (3.33%, 2/60) and 1:512 (3.33%, 2/60). The serological titer of 1:256 (0.0%) was negative for indirect immunofluorescence in all 60 mice evaluated with different concentrations of glycerol + tachyzoites. For IgG (chronic phase), the highest percentages were 42% (21/50), 40% (20/50) 12% (6/50) for titers 1:64, 1:32 and 1:16, respectively, with all DMSO concentrations tested. Regarding glycerol, the highest rate was found for the titer of 1:32 (36.66%, 22/60) and antibody titers of 1:16 and 1:64 had the same rates (21.66%, 13/60) for all concentrations evaluated (Table 3).

Analyzing statistical significance ($p \leq 0.05$) by Fisher's exact test for the IgM antibody, significant differences were found for DMSO concentrations of 5.0% and 7.5%, glycerol concentrations of 2.25%, 2.5%, 3.0%, 5.0%, 7.5% and 10.0% and samples with the absence of cryoprotectant ($p \leq 0.05$) regarding serological titers of 1:32 and 1:64 compared to the titers in the control group. Significant differences ($p \leq 0.05$) for the IgG antibody were only found with titers of 1:16 and 1:64 in the samples with 2.5% DMSO and 5.0% DMSO. Significant differences ($p \leq 0.05$) for the IgG antibody were only found with the titer of 1:16 in the samples with glycerol at concentrations of 2.25%, 3.0% and 5.0%. Samples with glycerol at concentrations of 2.5% and 10.0% had serological reciprocal titers at 1:16 and 1:64 and a significant difference ($p \leq 0.05$) was found for 10% glycerol at the titer of 1:32. In the group cryopreserved in liquid nitrogen (-196°C) without the addition of any cryoprotectant, we obtained results of 50% (5/10) for a titer 1:32 and 50% (5/10) for a titer of 1:64. All mice in the control group had negative serological results.

Groups	Scott-Knott (Means)
Glycerol 7.5%	6,262[a]
Glycerol 3.0%	4,746[a]
DMSO 8.0%	3,534[a]
DMSO 5.0%	3,324[a]
*Cryoprotectant free	3,108[a]
DMSO 2.5%	1,848[b]
DMSO 7.5%	1,758[b]
Glycerol 5.0%	1,608[b]
Glycerol 10.0%	1,404[b]
Glycerol 2.25%	1,398[b]
Glycerol 2.5%	1,398[b]
Controls	0.00[b]



2

Fig.2. Mean *Toxoplasma gondii* tachyzoites recovered and quantified (Scott-Knott test) with different concentrations of cryoprotectant (glycerol and DMSO).

Table 3. Quantification of anti-*Toxoplasma gondii* titre (IgM and IgG) in serum of mice experimentally infected with semen containing *T. gondii* tachyzoites + crioprotectors (DMSO and glycerol) in different concentrations and control groups

Experimental groups	Cryoprotectant (concentations)	Tachyzoites/ Inoculant dose	Mice serologic titer									
			Anti- *T. gondii* IgM Class						Anti- *T. gondii* IgG Class			
			16	32	64	128	256	512	16	32	64	128
I	DMSO 2.5%	1 x 10^6	2^A	4^A	4^A	0^A	0^A	0^A	2^{AB}	4^A	4^A	0^A
II	DMSO 5.0%	1 x 10^6	0^A	4^A	2^{AB}	2^A	0^A	2^A	4^A	4^A	2^{AB}	0^A
III	DMSO 7.5%	1 x 10^6	0^A	2^{AB}	4^A	2^A	2^A	0^A	0^B	4^A	5^A	0^A
IV	DMSO 8.0%	1 x 10^6	2^A	4^A	4^A	0^A	0^A	0^A	0^B	4^A	5^A	0^A
V	DMSO 10.0%	1 x 10^6	2^A	4^A	4^A	0^A	0^A	0^A	0^B	4^A	5^A	0^A
VI	Glycerol 2.25%	1 x 10^6	2^A	5^A	3^{BC}	0^A	0^A	0^A	2^{AB}	4^A	4^A	0^A
VII	Glycerol 2.5%	1 x 10^6	3^A	4^{AB}	3^{BC}	0^A	0^A	0^A	2^{AB}	4^A	4^{AB}	0^A
VIII	Glycerol 3.0%	1 x 10^6	1^A	4^{AB}	5^{AB}	0^A	0^A	0^A	2^{AB}	4^A	4^A	0^A
IX	Glycerol 5.0%	1 x 10^6	0^A	1^{BC}	5^{AB}	2^A	0^A	2^A	2^{AB}	4^A	4^A	0^A
X	Glycerol 7.5%	1 x 10^6	3^A	4^{AB}	3^{BC}	0^A	0^A	0^A	0^B	4^A	5^A	0^A
XI	Glycerol 10.0%	1 x 10^6	0^A	5^A	5^{AB}	0^A	0^A	0^A	5^{AB}	2^{AB}	0^B	0^A
XII	Crioprotector free	1 x 10^6	0^A	5^A	5^{AB}	0^A	0^A	0^A	2^{AB}	5^A	2^{AB}	1^A
XIII	DMSO 5.0% (control)	0	0^A	0^B	0^B	0^A	0^A	0^A	0^A	0^B	0^B	0^A
	Glycerol 3.0% (control)	0	0^A	0^B	0^B	0^A	0^A	0^A	0^A	0^B	0^B	0^A

^{A,B,C} Values followed by the same letter on the same row do not differ significantly at Fisher´s Exact test ($p \geq 0.05$).

DISCUSSION

The present work offers unprecedented results regarding the viability and infectivity of *Toxoplasma gondii* in different concentrations of cryoprotectants (DMSO and glycerol) used in animal reproduction. With most concentrations of glycerol and DMSO as well as samples without the addition of cryoprotectant exposed to a low temperature (-196°C in liquid nitrogen) for 24 hours, the quantification of viable tachyzoites was similar, with average survival of 7.6 days among the experimentally infected mice. The most important factor regards seminal parameters (vigor and motility), as the 2.5%, 3.0% and 5.0% glycerol groups had ideal parameters for bovine reproduction. Moreover, the 7.5%, 8.0%, 10% DMSO and 7.5% glycerol groups had close to appropriate parameters. This information is important when we consider the sexual transmission of toxoplasmosis and the small number of experimental trials in the literature involving the use of reproduction biotechnology (insemination artificial) for mammalian species and protozoan etiological agents, such as *T. gondii*.

The cryopreservation process causes physical, chemical and metabolic harm to sperm cells (Watson 2000). Adding the effects of all processes, an estimated 50% loss of sperm viability occurs from dilution to thawing (Watson 1995). The "Colégio Brasileiro de Reprodução Animal" (Brazilian College of Animal Husbandry) (CBRA 2013) determines sperm motility and vigor of at least 30% and a score of 3, respectively, for thawed bovine semen. In the present study, all thawed samples had lower values for both parameters (5.0% to 20% for motility and a score of 1 to 2 for vigor). Therefore, use in artificial insemination programs is considered inappropriate. Motility (%) and vigor (0-5) after the thawing of cryopreserved semen using DMSO were low with all concentrations of the cryoprotectant evaluated. However, thawed semen protected with glycerol had acceptable motility (4%) and vigor (35%), suggesting a good performance in terms of artificial insemination.

DMSO at concentrations ranging from 1 to 32% is widely used for the cryopreservation of mammalian semen and microorganisms (Sherman 1964, Zimmerman et al. 1964, O'Shea & Wales 1969, Hubálek 2003). In the specific case of *T. gondii*, Dumas (1974) found that the best concentration of DMSO for parasitic tachyzoite cryoprotection ranged from 5 to 10%, which is in agreement with the results of the present investigation.

Few studies have been conducted on the use of DMSO as a cryoprotectant for bovine semen. Snedeker & Gaunya (1970) studied the cryopreservation of bovine semen with different concentrations of DMSO alone or in combination with glycerol. Evaluating the isolated action of DMSO in homogenized whole milk diluent, the authors found semen motility of 11%, 20%, 24% and 18% after thawing using DMSO at concentrations of 2.0%, 4.0%, 6.0% and 8.0%, respectively. These figures are very similar to those obtained in the present study, as 20% semen motility was found for different concentrations of DMSO (7.0%, 8.0% and 10%). Although DMSO has been used with relative success for several animal species, its use in bovine semen cryopreservation is not as effective as glycerol due to its insufficient cryoprotective activity.

Strains of *T. gondii* are classified in terms of virulence based on the inoculation of laboratory animals with known doses of the parasite (Willians et al. 1978). Darde et al. (1988) found 100% lethality in Swiss mice inoculated with tachyzoites of the RH strain. Regardless of the dose, the longest survival period was found in mice inoculated with 10^4 tachyzoites (8.67 days on average). Derouin & Garin (1991) reported average survival of 6.5 days, with death occurring between the 6th and 8th DPI; however, the inoculation route was intraperitoneal. Inoculating 10^2 tachyzoites (RH strain) subcutaneously, Villard et al. (1995) reported average survival of 10.83 days, with the death of mice occurring between the 10th and 12th DPI. Regarding the dose of the inoculum, the present study was based on Scarpelli et al. (2009), who used a 5 x 10^6 tachyzoites injected directly into bovines through the subcutaneous route. As we used semen samples in mice

(intraperitoneal) with the virulent RH strain and did not know the effects of low temperature (-196ºC) on the replication of *T. gondii*, a dose of 1 x 10^6 tachyzoites was chosen.

The RH strain is highly pathogenic and is known to cause death in mice during the acute phase (Gravrilesco & Denkers 2001, Barragan & Sibley 2002). In the present study, average mortality of mice inoculated using the RH strain frozen for 24 hours at -196°C with cryoprotectants (different concentrations of DMSO and glycerol) was 7.6 to 9.2 days and clinical signs were observed beginning with the 6th DPI. All virulent of *T. gondii* strains behave differently from other genotypes, which have a slow onset and the formation of tissue cysts (Sevá et al. 2006).

The results of this study indicate that *T. gondii* maintained its infectivity in all contaminated samples, including in the absence of cryoprotectant. The maintenance of the viability and infectivity of *T. gondii* tachyzoites was confirmed in the bioassay, as all mice developed acute toxoplasmosis, which was diagnosed based on clinical signs. The virulence of the strain was confirmed by its lethality, with the death of 100% of the animals within 10 days after inoculation. It was not possible to calculate the infective dose effectively administered due to the loss of viable tachyzoites during the experimental procedures, such as semen dilution and thermal stress due to cooling, freezing and thawing.

All tachyzoite-inoculated mice exhibited seroconversion for IgM and IgG. However, different results have been reported in other studies (Camargo et al. 1991, Camargo 1996, Cunha et al. 2004, Guimarães et al. 2009, Costa-Silva & Pereira-Chioccola 2010). This may have occurred because the infective dose of tachyzoites inoculated in each animal was higher in the present investigation and different strains were used in other studies. Other differences regarding the virulence of strains likely occur due to successive passages of the parasite in the same animal species, such that the characteristic of virulence may be altered (Jacobs & Melton 1954). Based on these previous studies, the immunological response with production of anti-*T. gondii* antibodies (IgG) suggests a possible attenuation of tachyzoites during the cryopreservation process, which enabled greater antigenic stimulation in the infected animals.

Using semen cryopreserved with a commercial formulation containing 3% glycerol and contaminated with 10^7 tachyzoites of *T. gondii* frozen in liquid nitrogen and thawed, Consalter et al. (2017) found vigor and motility of 4% and 35%, respectively. To confirm viability, mice were inoculated with tachyzoites of *T. gondii* (1 x 10^6) + sperm (20 x 10^6) by the intraperitoneal route, all of which became ill and died within two weeks. Despite the difference between cattle and sheep semen, the same concentration of glycerol (3%) led to the same vigor and motility results in the present study after storage in liquid nitrogen and a similar period of sickness and death of the mice. Thus, this is likely a standard for the RH strain of *T. gondii*.

The sexual transmission of toxoplasmosis has been demonstrated in some species, such as sheep (Moraes et al. 2010, Lopes et al. 2013), goats (Santana et al. 2013) and rabbits (Liu et al. 2006). However, the maintenance of *T. gondii* tachyzoite viability in cryopreserved bull semen samples confirmed in the present study does not prove the infectivity of the parasite for bovine females through the vaginal route. Thus, future studies should be performed to evaluate whether tachyzoite-infected cryopreserved semen plays a role in *T. gondii* infection in cattle through the vaginal route.

CONCLUSION

Toxoplasma gondii tachyzoites remained viable and infectious in cryopreserved bovine semen containing DMSO and glycerol at all concentrations evaluated. Mice (Swiss strain) inoculated with cryopreserved semen contaminated with *T. gondii* tachyzoites exhibited clinical symptoms and seroconversion with varying titers of IgM and IgG.

Conflict of interest statement.- The authors declare having no conflicts of interest.

REFERENCES

Agca Y. & Critser J.K. 2002. Cryopreservation of spermatozoa in assisted reproduction. Semin. Reprod. Med. 20(1):15-23. <https://dx.doi.org/10.1055/s-2002-23516> <PMid:11941531>

Alton G.G., Jones L.M., Angus R.D. & Verger J.M. 1988. Techniques for the Brucellosis Laboratory. INRA Publications, Paris, p.63-129.

Barragan A. & Sibley L.D. 2002. Transepithelial migration of *Toxoplasma gondii* is linked to parasite motility and virulence. J. Exp. Med. 195(12):1625-1633. <https://dx.doi.org/10.1084/jem.20020258> <PMid:12070289>

Baruselli P.S., Ferreira R.M., Colli M.H.A., Elliff F.M., Sá Filho M.F., Vieira L. & Freitas B.G. 2017. Timed artificial insemination: current challenges and recent advances in reproductive efficiency in beef and dairy herds in Brazil. Anim. Reprod. 14(3):558-571. <https://dx.doi.org/10.21451/1984-3143-AR999>

Becker W.C., Senger P.L., Aalseth E.P. & Marshall C.E. 1977. Influence of glycerol levels, diluent and post-thaw temperature on motility and acrossomal maintenance in bovine semen frozen in plastic straws. J. Anim. Sci. 44(6):1067-1071. <https://dx.doi.org/10.2527/jas1977.4461067x> <PMid:873862>

Boni R. 2012. Ovum pick-up in cattle: a 25 yr retrospective analysis. Anim. Reprod. 9(3):362-369.

Bresciani K.D.S., Costa A.J., Toniollo G.H., Sabatini G.A., Morais F.R., Paulilo A.C. & Ferraudo A.S. 1999. Experimental toxoplasmosis in pregnant bitches. Vet. Parasitol. 86(2):143-145. <https://dx.doi.org/10.1016/S0304-4017(99)00136-3> <PMid:10496698>

Camargo M.E. 1964. Improved technique of indirect immunofluorescence for serological diagnosis of toxoplasmosis. Revta Inst. Med. Trop. São Paulo 6:117-118. <PMid:14177810>

Camargo M.E. 1996. Toxoplasmosis, p.165-174. In: Ferreira A.W. & Moraes S.L. (Eds), Diagnóstico Laboratorial das Principais Doenças Infecciosas e Auto-imunes. Guanabara Koogan, Rio de Janeiro.

Camargo M.E., Silva S.M., Leser P.G. & Granato C.H. 1991. Avidez de anticorpos IgG específicos como marcadores de infecção primária recente pelo *Toxoplasma gondii*. Revta Inst. Med. Trop. 33(3):213-218. <https://dx.doi.org/10.1590/S0036-46651991000300008>

Castelo T.S., Frota T.R. & Silva A.R. 2008. Considerações sobre a criopreservação do sêmen de caprinos. Acta Vet. Bras. 2(3):67-75. <https://dx.doi.org/10.21708/avb.2008.2.3.885>

CBRA. 2013. Manual para exame andrológico e avaliação de sêmen animal. 3ª ed. Colégio Brasileiro de Reprodução Animal, Belo Horizonte. 104p.

Centro Panamericano de Zoonoses. 1985. Salud Pública Veterinaria. Ministerio de Salud y Acción Social, Organización Panamericana de la Salud, Organización Mundial de la Salud, Argentina, p.193-204.

Conrad P., Barr B.C., Sverlow K.W., Anderson M., Daft B., Kinde H., Dubey J.P., Munson L. & Ardans A. 1993. *In vitro* isolation and characterization of a

Neospora sp. from aborted bovine foetuses. Parasitology 106(Pt 3):239-249. <https://dx.doi.org/10.1017/S0031182000075065> <PMid:8488061>

Consalter A., Silva A.F., Frazao-Teixeira E., Matos L.F., Oliveira F.C.R., Leite J.S., Silva F.B.F. & Ferreira A.M.R. 2017. *Toxoplasma gondii* transmission by artificial in sheep with experimentally contaminated frozen semen. Theriogenology 90:169-174. <https://dx.doi.org/10.1016/j.theriogenology.2016.12.004> <PMid:28166964>

Costa A.J., Araujo F.G., Costa J.O., Lima J.D., Nascimento E. 1977. Experimental infection of bovines with oocysts of *Toxoplasma gondii*. J. Parasitol. 63(2):212-218. <https://dx.doi.org/10.2307/3280042> <PMid:558305>

Costa G.H.N., Costa A.J., Lopes W.D.Z., Bresciani K.D.S., Santos T.R., Esper C.R. & Santana A.E. 2011. *Toxoplasma gondii*: infection natural congenital in cattle and an experimental inoculation of gestating cows with oocysts. Exp. Parasitol. 127(1):277-281. <https://dx.doi.org/10.1016/j.exppara.2010.08.005> <PMid:20736009>

Costa-Silva T.A. & Pereira-Chioccola V.L. 2010. Fase aguda da infecção por *Toxoplasma gondii*: avaliação do parasitismo sanguíneo e resposta humoral em camundongos isogênicos AS/n. Scientia Medica 20(1):88-92.

Cunha E.L.P., Silva R.C., Da Silva A.V. & Langoni H. 2004. The protective effects of Baypamun® HK in mice experimentally infected with *Toxoplasma gondii*. Braz. J. Vet. Res. Anim. Sci. 41(3):169-172. <https://dx.doi.org/10.1590/S1413-95962004000300004>

Darde M.L., Bouteille B. & Pestre-Alexandre M. 1988. Izoenzymic characterization of seven strains of *Toxoplasma gondii* by electrofocussing in polyacrilamid gels. Am. J. Trop. Med. Hyg. 39(6):551-558. <https://dx.doi.org/10.4269/ajtmh.1988.39.551> <PMid:2462794>

Derouin F. & Garin Y.J.F. 1991. *Toxoplasma gondii*: blood and tissue kinetics during acute and chronic infections in mice. Exp. Parasitol. 73(4):460-468. <https://dx.doi.org/10.1016/0014-4894(91)90070-d> <PMid:1959573>

Duarte J.M.B. & Garcia J.M. 1997. Tecnologia da reprodução para propagação e conservação de espécies ameaçadas de extinção, p.228-238. In: Duarte J.M.B. (Ed.), Biologia e Conservação de Cervídeos Sul-americanos: *Blastocerus*, *Ozotoceros* e *Mazama.* FUNEP, Jaboticabal.

Dubey J.P. & Beattie C.P. 1988. Toxoplasmosis of animals and man. CRC Press, Boca Raton, p.1-220.

Dubey J.P. & Lindsay D.S. 1996. A review of *Neospora caninum* and neosporosis. Vet. Parasitol. 67, 1–59. <https://doi.org/10.1016/S0304-4017(96)01035-7>

Dubey J.P., Hotea I., Olariu T.R., Jones J.L. & Darabus G. 2013. Epidemiological review of Toxoplasmosis in humans and animals in Romania. Parasitology 141(3):311-325. <https://dx.doi.org/10.1017/S0031182013001509> <PMid:24553077>

Dumas N. 1974. Conservation aux basses temperatures de *Toxoplasma gondii* (Nicolle et Manceaux, 1909): action du dimeethyl-sulfoxyde. Ann. Parasitol. Hum. Comp. 49(1):1-40. <https://dx.doi.org/10.1051/parasite/1974491001>

Fahy G.M. 1986. The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology 23(1):1-13. <https://dx.doi.org/10.1016/0011-2240(86)90013-1> <PMid:3956226>

Fialho C.G., Teixeira M.C. & De Araújo F.A.P. 2009. Toxoplasmose animal no Brasil. Acta Scient. Vet. 37(1):1-23. <https://dx.doi.org/10.22456/1679-9216.16180>

Fickel J., Wagener A. & Ludwig A. 2007. Semen cryopreservation and the conservation of endangered species. Eur. J. Wildl. Res. 53:81-89. <https://dx.doi.org/10.1007/s10344-007-0089-z>

Figliuolo L.P.C., Rodrigues A.A.R., Viana R.B., Aguiar D.M., Kasai N. & Gennari S.M. 2004. Prevalence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies in goat from São Paulo State, Brazil. Small Ruminant Res. 55(1/3):29-32. <https://dx.doi.org/10.1016/j.smallrumres.2003.12.013>

Fontgibell A. & Vidal A.F. 1998. Primeros resultados en el estudio de los efectos de la congelación de semen de gallo en tres razas catalanas. Arch. Zootec. 47(178/179):335-341.

Gravrilesco L.C. & Denkers E.Y. 2001. IFN-gama overproduction and high level apoptosis are associated with high but not low virulence *Toxoplasma gondii* infection. J. Immunol. 167(2):902-909. <https://dx.doi.org/10.4049/jimmunol.167.2.902> <PMid:11441097>

Guimarães L.R., Barbaresco A.A. & Castro A.M. 2009. Perfil sorológico de camundongos cronicamente infectados com a cepa cistogênica ME-49 de *Toxoplasma gondii* antes e após terapêutica específica. Revta Patol. Trop. 38(4):261-266. <https://dx.doi.org/10.5216/rpt.v38i4.8589>

Homan W.L., Vercammen M., De Braekeleer J. & Verschueren H. 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int. J. Parasitol. 30(1):69-75. <https://dx.doi.org/10.1016/s0020-7519(99)00170-8> <PMid:10675747>

Hubálek Z. 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology 46(3):205-229. <https://dx.doi.org/10.1016/S0011-2240(03)00046-4> <PMid:12818211>

Jacobs L. & Melton M.L. 1954. Modification in virulence of a strain of *Toxoplasma gondii* by passage in various host. Am. J. Trop. Med. Hyg. 3(3):447-457. <https://dx.doi.org/10.4269/ajtmh.1954.3.447> <PMid:13158764>

Kashiwazaki N., Okuda Y., Seita Y., Hisamatsu S., Soroki S., Shino M., Masaoka T. & Inomata T. 2006. Comparison of glycerol, lactamide and dimethyl sulfoxide as cryoprotectants of Japanese white rabbit spermatozoa. J. Reprod. Develop. 52(4):511-516. <https://dx.doi.org/10.1262/jrd.18008> <PMid:16679717>

Liu S.-G., Qin C., Yao Z.-J. & Wang D. 2006. Study on the transmission of *Toxoplasma gondii* by semen in rabbits. Chinese J. Parasitol. Parasit. Dis. 24(3):166-170. <PMid:17094613>

Lopes W.D.Z., Rodriguez J.D., Souza F.A., Santos T.R., Santos R.S., Rosanese W.M., Lopes W.R.Z., Sakamoto C.A. & Costa A.J. 2013. Sexual transmission of *Toxoplasma gondii* in sheep. Vet. Parasitol. 195(1/2):47-56. <https://dx.doi.org/10.1016/j.vetpar.2012.12.056> <PMid:23384578>

Lovelock J.E. & Bishop M.W.H. 1959. Prevention of freezing damage to living cells by dimethylsulphoxide. Nature 183(4672):1394-1395. <https://dx.doi.org/10.1038/1831394a0><PMid:13657132>

Mascarenhas H. & Gomes W.V. 1950. Contribuição ao Estudo da Eletroejaculação em Bovinos: novo tipo de elétrodo bipolar e técnica de sua aplicação. Vol.8. Instituto de Zootecnia, Rio de Janeiro. 30p.

Mello V. 1910. Un cas de toxoplasmose du chien observé à Turin. Bulletin de la Societe de Pathologie 28:359-363.

Mineo J.R., Camargo M.E. & Ferreira A.W. 1980. Enzyme-linked immunosorbent assay for antibodies to *Toxoplasma gondii* polysaccharides in human toxoplasmosis. Infect. Immun. 27(2):283-287. <https://dx.doi.org/10.1128/iai.27.2.283-287.1980**>** <PMid:7380534>

Mitsuka R., Beckner Da Silva A.C., Navarro I.T., Breganó J.W. & Vidotto O. 1998. *Toxoplasma gondii*: I. Avaliação da virulência de oito amostras. Braz. J. Vet. Res. Anim. Sci. 35(1):29-31. <https://dx.doi.org/10.1590/S1413-95961998000100005>

Moraes E.P.B.X., Batista A.M., Faria E.B., Freire R.L., Freitas A.C., Silva M.A.R., Braga V.A. & Mota R.A. 2010. Experimental infection by *Toxoplasma gondii* using contaminated semen containing different doses of tachyzoites in sheep. Vet. Parasitol. 170(3/4):318-322. <https://dx.doi.org/10.1016/j.vetpar.2010.02.017> <PMid:20227187>

Murgas L.D.S., Miliorini A.B., Freitas R.T.F. & Pereira G.J.M. 2007. Criopreservação do sêmen de curimba (*Prochilodus lineatus*) mediante adição de diferentes diluidores, ativadores e crioprotetores. Revta Bras. Zootec. 36(3):526-531. <https://dx.doi.org/10.1590/S1516-35982007000300002>

O'Shea T. & Wales R.G. 1969. Further studies of the deep freezing of rabbit spermatozoa in reconstituted skim milk powder. Austr. J. Biol. Sic. 22(3):709-720. <https://dx.doi.org/10.1071/BI9690709> <PMid:5801283>

Polge C., Smith A.U. & Parkers A.S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164(4172):666. <https://dx.doi.org/10.1038/164666a0> <PMid:18143360>

Quinn P.J. & Mccraw B.M. 1972. Current status of *Toxoplasma* and toxoplasmosis: a review. Can. Vet. J. 13(11):247-262. <PMid:4567530>

Ribeiro R.I.M.A. & Godinho H.P. 2003. Criopreservação do sêmen testicular do teleósteo piau-açu *Leporinus macrocephalus*. Arq. Bras. Med. Vet. Zootec. 55(1):75-79. <https://dx.doi.org/10.1590/S0102-09352003000100011>

Rodgers J.C., Bird S.L., Larson J.E., DiLorenzo N., Dahlen C.R., DiCostanzo A. & Lam G.C. 2012. An economic evaluation of estrous synchronization and timed artificial insemination in suckled beef cows. J. Anim. Sci. 90(11): 4055-4062. <https://dx.doi.org/10.2527/jas.2011-4836> <PMid:22585820>

Sabin A.B. 1941. Toxoplasmic encephalitis in children. J. Am. Med. Assoc.116(9):801-807. <https://dx.doi.org/10.1001/jama.1941.02820090001001>

Sanches E.G. & Oliveira I.R. & Serralheiro P.C.S. 2008. Crioconservação do sêmen da garoupa-verdadeira *Epinephelus marginatus*. Bioikos 22(2):81-90.

Santana L.F., Rossi G.A.M., Gaspar R.C., Pinto V.M.R., Oliveira G.P. & Costa A.J. 2013. Evidence of sexual transmission of *Toxoplasma gondii* in goats. Small Rumin. Res. 115(1/3):130-133. <https://dx.doi.org/10.1016/j.smallrumres.2013.08.008>

Sawadogo P., Hafid J., Bellete B., Sung R.T.M., Chakdi M., Flori P., Raberin H., Hamouni, I.B., Chait A. & Dalal A. 2005. Soroprevalence of *T. gondii* in sheep from Marrakech, Morocco. Vet. Parasitol. 130(1/2):89-92. <https://dx.doi.org/10.1016/j.vetpar.2005.03.025> <PMid:15893074>

Scarpelli L., Lopes W.D.Z., Migani M., Bresciani K.D.S. & Costa A.J. 2009. *Toxoplasma gondii* in experimentally infected *Bos taurus* and *Bos indicus* semen and tissues. Pesq. Vet. Bras. 29(1):59-64. <https://dx.doi.org/10.1590/S0100-736X2009000100009>

Sevá A.P., Silva R.C., Silva A.V., Castro A.P.B., Menozzi B.D. & Langoni H. 2006. Avaliação da virulência de cepas de *Toxoplasma gondii*, em camundongos, isoladas de cães com sinais neurológicos, em Botucatu, SP. Vet. Zootec. 13(1):33-43.

Sherman J.K. 1964. Dimethyl sulfoxide as a protective agent during freezing and thawing of human spermatozoa. Exper. Biol. Med. 117(1). <https://dx.doi.org/10.3181/00379727-117-29552> <PMid:14223287>

Snedeker W.H. & Gaunya W.S. 1970. Dimethyl sulfoxide as a cryoprotective agent for freezing bovine semen. J. Anim. Sci. 30(6):953-956. <https://dx.doi.org/10.2527/jas1970.306953x> <PMid:5463531>

Soares H.S., Ahid S.M.M., Bezerra A.C.D.S., Pena H.F.J., Dias R.A. & Gennari S.M. 2009. Prevalence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies in sheep from Mossoró, Rio Grande do Norte, Brazil. Vet. Parasitol. 160(3/4):211-214. <https://dx.doi.org/10.1016/j.vetpar.2008.10.102> <PMid:19091473>

Teale A.J., Blewett D.A., Miller J.K. & Buxton D. 1982. Experimentally induced toxoplasmosis in young rams: The clinical syndrome and semen secretion of toxoplasma. Vet. Rec. 111(3):53-55. <https://dx.doi.org/10.1136/vr.111.3.53> <PMid:7123822>

Tenter A.M., Heckeroth A.R. & Weiss L.M. 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30(12/13):1217-1258. <https://dx.doi.org/10.1016/S0020-7519(00)00124-7> <PMid:11113252>

Villard O., Candolfi E., Despringre J.L., Deroum F., Marcellin L., Viville S. & Kien T. 1995. Protective effectof low doses of an anti-IL-4 monoclonal antibody in a murme model of acute toxoplasmosis. Parasite Immunul. 17(5):233-326. <https://dx.doi.org/10.1111/j.1365-3024.1995.tb01020.x> <PMid:7675509>

Vishwanath R. 2003. Artificial insemination: the state of the art. Theriogenology. 59(2):571-584. <https://dx.doi.org/10.1016/S0093-691X(02)01241-4>

Watson P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Develop. 7(4):871-891. <https://dx.doi.org/10.1071/rd9950871> <PMid:8711221>

Watson P.F. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60/61:481-492. <https://dx.doi.org/10.1016/s0378-4320(00)00099-3> <PMid:10844218>

Willians D.M., Grumet F.C. & Remington J.S. 1978. Genetic control of murine resistance to *Toxoplasma gondii*. Infect. Immun. 19(2):416-420. <https://dx.doi.org/10.1128/iai.19.2.416-420.1978> <PMid:631881>