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In vitro viability of canine spermatozoa frozen in tris-fructose-citric acid extender with ethylene glycol

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Viabilidade *in vitro* do espermatozóide canino congelado em diluidor tris-frutose-ácidocítrico com etileno glicol

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SUMMARY

The use of frozen semen has become increasingly popular in canine artificial insemination, stimulating research to improve freezing methods and extenders. In this study, was compared the final concentration of 5% ethylene glycol used in 3 equilibration protocols. In Method I, one part of semen was added to two parts of tris-fructose-citric acid with 7.5% ethylene glycol (Extender 2) and frozen without cooling. In Method II, one part of semen was added to two parts of tris-fructose-citric acid with 7.5% ethylene glycol (Extender 2) and cooled at 5°C for 1 hour before freezing. In Method III, one part of semen was added to one part of tris-fructose-citric without ethylene glycol (Extender 1) and cooled at 5°C for 1 hour. Just after this period, one part of 5°C cooled tris-fructose-citric acid containing 15% ethylene glycol (Extender 3) was added to the previous extended semen, maintained at 5°C for one additional hour and frozen. For freezing in each procedure semen was packaged in 0.5 ml plastic straws and placed for 20 minutes in vapor 5 cm above liquid nitrogen and then lowered into the liquid nitrogen and stored. All samples were thawed by immersion for 30 sec in a 37°C water bath and immediately evaluated. Progressive motility was best after Method III (63%), which was not different from fresh semen (94%) but was better ($p<0.05$) than after Method II (25%) or Method I (6%). Forward velocity was again best after Method III (2.9) comparable to fresh semen (4.6) and Method II (2.5), but better ($p<0.05$) than Method I (1.7). Morphological abnormalities were lowest after Method III, but were not significantly different from Methods II or I. Most common abnormalities were detached heads and bent, hairpin and coiled tails. It was concluded that slow, step-wise equilibration in tris-fructose-citrate extender followed by extender with ethylene glycol before freezing seems to produce the best progressive motility and forward velocity in frozen-thawed canine spermatozoa.

UNITERMS: Ethylene glycol; Cryoprotectants; Freezing; Semen; Dog.

INTRODUCTION

The use of frozen semen is indicated as an alternative to natural breeding in reproductive management and for the preservation of genetic material from dogs, as well as for reducing the need to travel for the stud and bitch.

Semen freezing is commonly performed by using tris-fructose-citric acid extender and 4% glycerol as a cryoprotectant[1,2,4,12,20,23,24]. However, conception rates using frozen semen with glycerol were around 70%[13]. Spermatozoa freezing procedures decrease fertilization capacity, due to the reduction of progressive motility and velocity of forward progression and the increase in sperm morphological abnormalities after thawing. High concentrations of cryoprotectant can interfere with fertilization capacity, although without killing the spermatozoa[4]. Therefore, it is important and necessary to research freezing techniques and extenders, which diminish harmful effects to spermatozoa and, consequently, increase the fertility rates.

Cryobiology has been extensively studied in other

species, such as bovine and equine, but less investigated in canine. Ethylene glycol is classified as an intracellular cryoprotectant with a molecular weight lower than glycerol[15,19]. Consequently, ethylene glycol can readily cross cell membranes and hence penetrate and leave the cell faster than glycerol. This cryoprotectant is frequently and satisfactorily used as a bovine, caprine and ovine frozen embryo protectant, as well as a spermatozoa cryoprotectant for avian frozen semen[10,14]. Bovine and ovine embryos have greater permeability to ethylene glycol than other cryoprotectants[22].

Progressive motility is used to evaluate the success of freezing procedures, providing inferences about the stud fertility. Therefore, it is convenient to appraise semen progressive motility after thawing to estimate the viability of the spermatozoa[8,16]. Dobrinski *et al.*[5] referred to the velocity of the forward progression as a parameter to evaluate canine spermatozoa after thawing. However, this parameter is not widely used.

The purpose of this study was to compare three different freezing procedures for canine spermatozoa using tris-fructose-citric acid with a 5% final concentration of ethylene glycol, considering progressive motility, velocity of forward progression and morphological abnormalities.

Table 1

Means and standard deviation of spermatozoa progressive motility (%) in ejaculates of ten dogs, before and after freezing by methods I, II and III. São Paulo, 1996.

		Post thaw semen		
Dog	Fresh Semen	Method I	Method II	Method III
1	75.00 (±7.07)	0 (±0)	5.00 (±0)	60.00 (±14.14)
2	90.00 (±0)	1.00 (±0)	17.50 (±17.68)	65.00 (±21.21)
3	95.00 (±0)	0 (±0)	62.50 (±17.68)	55.00 (±7.07)
4	95.00 (±0)	0.50 (±0.71)	72.50 (±3.53)	75.00 (±7.07)
5	92.50 (±3.53)	5.00 (±0)	35.00 (±21.21)	75.00 (±7.07)
6	87.50 (±10.61)	0.50 (±0.71)	22.50 (±24.75)	25.00 (±7.07)
7	95.00 (±0)	1.00 (±0)	10.00 (±14.14)	60.00 (±14.14)
8	82.50 (±17.68)	30.50 (±41.72)	40.00 (±49.50)	65.00 (±21.21)
9	92.50 (±3.53)	0.50 (±0.71)	7.50 (±10.61)	57.50 (±24.75)
10	92.50 (±3.53)	2.50 (±3.53)	7.50 (±3.53)	37.50 (±3.53)
Total	94.30 (±6.50)[a]	6.00 (±9.38)[c]	24.90 (±24.03)[b]	62.90 (±15.63)[a]

[b; c] -Columns with different superscripts differ $p<0.05$.

Table 2

Means and standard deviation of the velocity of forward progression of spermatozoa (0-5) in ejaculates of ten dogs, before and after freezing by methods I, II and III. São Paulo, 1996.

		Post thaw semen		
Dog	Fresh Semen	Method I	Method II	Method III
1	3.00 (±0)	0.00 (±0)	2.00 (±0)	2.50 (±0.71)
2	4.00 (±0)	2.50 (±0.71)	3.00 (±0)	3.00 (±0)
3	5.00 (±0)	0.00 (±0)	3.00 (±0)	3.00 (±0)
4	5.00 (±0)	1.00 (±1.41)	3.50 (±0.71)	3.50 (±0.71))
5	4.50 (±0.71)	2.50 (±0.71)	3.00 (±0)	3.00 (±0)
6	4.50 (±0.71)	1.50 (±2.12)	2.00 (±0)	1.50 (±0.71)
7	5.00 (±0)	1.00 (±0)	1.50 (±2.12)	3.00 (±0)
8	4.00 (±1.41)	2.50 (±0.71)	3.00 (±1.41)	3.00 (±1.41)
9	5.00 (±0)	0.50 (±0.71)	1.50 (±2.12)	3.00 (±0)
10	5.00 (±0)	1.00 (±1.41)	3.00 (±0)	3.00 (±0)
Total	4.60 (±0.67)[a]	1.70 (±0.98)[b]	2.50 (±0.72)[a]	2.90 (±0.53)[a]

[a; b] Columns with different superscript differ $p<0.05$.

MATERIAL AND METHOD

Experimental Design and Semen Collection

Ten sexual mature dogs of different breeds from a single kennel were used; one Bull Terrier, one Schnauzer, one Beagle, one Fila Brasileiro, one Mastiff and five Great Danes. The age of the animals ranged from 1 to 8 years and they were all housed in controlled environment, receiving commercial dog food.

Twenty semen samples were collected, two samples from each animal, at intervals of at least 48 hours between each collection. Only the sperm-rich fraction was collected by digital stimulation[6,11,13]. Depending on the male's libido, the presence of bitches was used to insure the sperm collection. All collections were done by the same person, using a glass funnel attached to a calibrated glass tube[11] plunged into water at 37°C in a baby-bottle.

Semen examination

Immediately after the collection, macroscopic parameters as color and volume of each ejaculate were appraised. Progressive motility (0 - 100%) and velocity of forward progression (0 - 5) were evaluated with the aid of an optical microscope, using a semen drop between a glass slide and a cover glass, both at 37°C[5,18]. Semen was also evaluated for sperm morphology. The ten dogs used in this experiment produced ejaculates with progressive motility greater than 70% and velocity of forward progression above 3[8].

Extenders

Extender 1: tris-fructose-citric acid (3.187% hydroximethyl-methylamine, 1.781% citric acid, 1.316% fructose, 20% egg yolk, and 100 ml distilled water).

Extender 2: tris-fructose-citric acid (3.187% hydroximethyl-methylamine, 1.781% citric acid, 1.316% fructose, 20% egg yolk, and 100 ml distilled water) and 7.5% of ethylene glycol.

Extender 3: tris-fructose-citric acid (3.187% hydroximethyl-methylamine, 1.781% citric acid, 1.316% fructose, 20% egg yolk, and 100 ml distilled water) and 15.0% of ethylene glycol.

Freezing procedures

The sperm-rich fraction from each dog was divided into 3 equal aliquots. Each of the three aliquots were extended and cryopreserved using one of the three different methods described below:

Method I: one part of semen was added to two parts of extender 2 at 37°C to reach 5% in final concentration of the ethylene glycol, then packaged into 0.5 ml plastic straws and immediately frozen and stored in liquid nitrogen.

Method II: one part of semen was added to two parts of extender 2 at 37°C to reach 5% in final concentration of the ethylene glycol. The extended semen was cooled during one hour to reach a uniform 5°C temperature and then packaged into 0.5 ml plastic straws, frozen and stored in liquid nitrogen.

Method III: one part of semen was added to one part of extender 1 (tris-fructose-citric without ethylene glycol) at 37°C and cooled during one hour to reach a uniform 5°C temperature. After this period, one part of extender 3 was added to the previous extended semen at 5°C for a final ethylene glycol concentration of 5%. The diluted semen was kept at this temperature for one additional hour to allow for the equilibration of the spermatozoa in the ethylene glycol. Then, the semen was packaged into 0.5 ml plastic straws, frozen and stored in liquid nitrogen.

The freezing procedure was the same for all three methods, the straws were exposed to nitrogen vapor at 5 cm of the liquid column for 20 minutes and plunged into liquid nitrogen[4,23].

Post-thaw evaluation

Thawing process was performed by immersion for 30 seconds in a 37°C water bath[23]. The semen was immediately evaluated in a pre-warmed slide and cover glass for progressive motility and velocity of forward progression. Semen from each method was added to a saline formaldehyde solution (12.5% of formaldehyde, 30% of NaCl and 20% of a buffer solution) to appraise sperm morphology, with the aid of a x1,000 magnification differential interference contrast microscope. Sperm morphological abnormalities were classified as major (any abnormality related to infertility or pathological conditions in the testis or epididymides) and minor (any abnormality recognized as less important), according to Blom[3] and Rao[17].

Statistical analysis

Data was analyzed according to the Tukey test by multiple comparisons of means. A 5% statistical level of significance was used to compare means and analysis of variance[21]. The kind of morphological abnormalities was examined using a non-parametrical test (Friedman two-way test).

RESULTS

The best results for progressive motility were obtained from fresh semen (94.30%) and from method III (62.90%), this does not present significant difference. On the other hand, methods I (6.00%) and II (24.90%) showed significantly lower progressive motility compared to method III (62.90%). Progressive motility after thawing in the three methods varied from 0 to 75% between the dogs, and even among collections from the same animal (Tab. 1).

Velocity of forward progression of spermatozoa from fresh semen (4.60) and from post-thaw semen of methods II (2.50) and III (2.90) did not show significant difference. Post-thaw semen of method I showed the lowest velocity of forward progression (1.70). (Tab. 2).

Regarding sperm morphological abnormalities in the post-thaw semen, the best results for major (Tab. 3) and minor (Tab. 4) abnormalities were noticed in method III. These results however did not differ significantly from method I and method II.

The types of abnormality more frequently found after semen thawing were detached heads and tail alterations, specially, bent, hairpin and coiled tails. The defects of bent tail, coiled tail and detached head were not statistically different among the methods and the fresh semen. On the other hand, all methods significantly increased hairpin tail defect, compared to the fresh semen, even though no difference was observed between methods I, II and III (Tab.5).

DISCUSSION

Progressive motility was used as the parameter to appraise ethylene glycol effectiveness as a cryoprotectant in canine frozen semen[8,16]. There is controversy among researchers about progressive motility as a parameter for spermatozoa viability analyses and, therefore, fertilization capacity. For instance, Yubi *et al.*[25] mentioned studies in the bull and ram which demonstrated that there is no relationship between low progressive motility and low fertility rates. For this reason, velocity of forward progression and sperm morphological abnormalities were also evaluated in this experiment.

The 20 ejaculates used in this experiment followed seminal characteristic patterns for freezing semen, which requires a progressive motility superior than 70% and a velocity of forward progression above 3[8].

The best canine semen freezing procedure was method III, according to results of progressive motility and velocity of forward progression. Results indicated that ethylene glycol was effective in protecting the spermatozoa through the freezing process, although the post-thaw seminal parameters decreased.

Considering sperm morphological abnormalities, the freezing process increased major abnormalities, although this alteration was not significant, which permitted to infer that there was no significant influence of the extenders and cryoprotectant on sperm morphology. However, it is important to emphasize that this work did not utilize a method to evaluate

Table 4

Means and standard deviation of minor sperm morphological abnormalities (%) in ejaculates of ten dogs, before and after freezing by methods I, II and III. São Paulo, 1996.

Dog	Fresh Semen	Post thaw semen		
		Method I	Method II	Method III
1	9.50 (±3.53)	6.00 (±2.83)	5.50 (±0.71)	6.00 (±1.41)
2	12.00 (±5.66)	7.50 (±3.53)	12.50 (±4.95)	8.50 (±6.36)
3	5.50 (±0.71)	1.50 (±0.71)	3.50 (±2.12)	2.00 (±1.41)
4	3.50 (±0.71)	1.50 (±0.71)	3.50 (±2.12)	0.50 (±0.71)
5	20.00 (±2.83)	8.50 (±2.12)	13.50 (±9.19)	10.50 (±2.12)
6	8.50 (±0.71)	1.00 (±0)	3.00 (±1.41)	7.00 (±0)
7	1.00 (±0)	4.50 (±2.12)	2.00 (±1.41)	0 (±0)
8	12.50 (±4.95)	5.00 (±4.24)	8.50 (±0.71)	3.00 (±1.41)
9	9.50 (±2.12)	8.00 (±1.41)	2.50 (±3.53)	6.50 (±2.12)
10	7.50 (±2.12)	6.00 (±5.66)	4.50 (±3.53)	6.50 (±4.95)
Total	8.90 (±5.29)[a]	4.90 (±3.04)[a]	5.90 (±4.17)[a]	5.00 (±3.50)[a]

[a] Columns with the same superscript do not differ $p<0.05$.

Table 5

Means and standard deviation of hairpin tail defect of spermatozoa (%) in ejaculates of ten dogs, before and after freezing by methods I, II and III. São Paulo, 1996.

		Post thaw semen		
Dog	Fresh Semen	Method I	Method II	Method III
1	3.50 (±0.71)	11.50 (±0.71)	13.00 (±1.41)	3.50 (±5.66)
2	7.00 (±4.24)	24.00 (±4.24)	7.50 (±7.78)	9.50 (±0.71)
3	11.50 (±4.95)	23.5 (±3.53)	20.50 (±0.71)	10.50 (±0.71)
4	3.00 (±2.83)	12.50 (±4.95)	6.50 (±9.19)	17.00 (±11.31)
5	1.00 (±1.41)	12.50 (±10.61)	14.00 (±12.73)	13.50 (±12.02)
6	24.00 (±9.90)	27.00 (±11.31)	25.00 (±0)	29.00 (±4.24)
7	1.50 (±2.12)	7.50 (±2.12)	15.50 (±9.19)	8.00 (±0)
8	16.50 (±2.12)	17.00 (±7.07)	13.00 (±2.83)	11.50 (±2.12)
9	1.50 (±2.12)	20.50 (±3.52)	28.50 (±12.02)	18.50 (±6.36)
10	2.50 (±0.71)	18.50 (±2.12)	12.00 (±1.41)	9.50 (±2.12)
Total	7.10 (±8.32)[a]	16.95 (±7.61)[b]	14.45 (±8.86)[b]	13.55 (±7.25)[b]

[a, b] Columns with different superscript differ $p<0.05$.

the membrane integrity of the sperm as a manner to assess plasmatic membrane and acrossomic lesions by staining the spermatozoa with carboxyfluorescein diacetate and propidium iodide[7]. Therefore, it is not known if the cryoprotectant and extender could have promoted damage to the sperm membranes. All three methods significantly increased hairpin defect, compared to the fresh semen, therefore, there was no difference in freezing canine spermatozoa with any of the three methods, considering the kind of morphological abnormalities.

Some ejaculates showed fewer sperm abnormalities after thawing, compared to the initial percentage of the fresh semen. This can be explained by the number of cytoplasmic droplets in fresh semen, which broke up through the freezing process and, therefore, did not appear in post-thaw semen.

Regarding methods I and II, it was believed that there was not enough time for cryoprotectant equilibration at 37°C (method I), as well as at lower temperatures (method II). In addition, Yubi *et al.*[25] indicates that when adding semen to an extender, an equilibration period of at least two hours is needed. Therefore, method III is ideal for freezing semen with ethylene glycol, since this method includes a period of 1 hour at 5°C for semen stabilization in extender without cryoprotectant (extender 1) and 1 hour in extender with cryoprotectant (extender 2) for equilibration. According to observations from Dobrinski *et al.*[5], slower freezing procedures are more beneficial to spermatozoa viability than faster procedures. Pursuant to this data, method III confirmed to be a better canine semen freezing method.

Nevertheless, progressive motility in the experiment III did not suffer an abrupt decrease after thawing, so we can affirm that ethylene glycol is more efficient in protecting canine spermatozoa against freezing effects. As ethylene glycol can move easily across the cell membrane due to the high permeability coefficient, the damage effects of dehydration and rehydration during the freezing and thawing procedures could have been partially avoided. During the freezing process, the spermatozoa dehydrates in contact with the hipertonic cryoprotectant solution, however, the cell rapidly returns to normal size due to the facility of this cryoprotectant in crossing through the cell membrane. Through the thawing process, the spermatozoa rapidly rehydrates and returns to normal size because ethylene glycol leaves the cell readily. Consequently, spermatozoa damaging during the freezing and thawing processes could have been minimized by the use of ethylene glycol.

This research demonstrated that ethylene glycol was efficient for spermatozoa survival through the freezing process in method III. Although the freezing procedure caused increased tail and head defects, it is not known whether this can interfere in fertilization. The fertility success of frozen semen in ethylene glycol can only be inferred by future studies using artificial insemination.

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RESUMO

A utilização de sêmen congelado tornou-se extremamente comum na prática da inseminação artificial em cães, estimulando a pesquisa de métodos de congelação e diluidores. Neste estudo foram avaliadas as propriedades criopreservativas do etileno glicol na concentração final de 5%, utilizado em 3 diferentes protocolos de congelação. No Método I, uma parte de sêmen foi adicionada a duas partes de tris-frutose-ácido cítrico, contendo 7,5% de etileno glicol e congelado sem prévia refrigeração. No Método II, uma parte de sêmen foi adicionada a duas partes de tris-frutose-ácido cítrico contendo 7,5% de etileno glicol e resfrigerado até 5°C por 1 hora antes da congelação. No Método III, uma parte de sêmen foi adicionada a uma parte de tris-frutose-ácido cítrico sem etileno glicol e refrigerado até 5°C por 1 hora, sendo em seguida adicionada uma parte de tris-frutose-ácido cítrico, previamente refrigerado até 5°C, contendo 15% de etileno glicol, mantido a 5°C por mais 1 hora e congelado. Para a congelação em cada método, o sêmen foi envasado em palhetas plásticas de 0,5 ml, colocadas em vapor de nitrogênio a 5 cm da coluna líquida por 20 minutos, mergulhadas no nitrogênio líquido e armazenadas. Todas as amostras foram descongeladas em banho-maria a 37°C durante 30 segundos e imediatamente avaliadas. O Método III não apresentou decréscimo significante da motilidade retilínea progressiva após a descongelação e apresentou os melhores resultados para o vigor espermático, comparando-se aos demais métodos. Entretanto, os 3 métodos não apresentaram diferença significativa com relação aos defeitos espermáticos pós-descongelação. Os tipos de defeitos mais freqüentemente encontrados foram cabeça solta normal, cauda dobrada, fortemente dobrada e fortemente enrolada. Foi possível concluir que o tempo de equilíbrio maior no diluidor tris-frutose-ácido cítrico seguido pela diluição em etileno glicol antes da congelação parece produzir para os espermatozóides de cães a melhor motilidade retilínea progressiva e vigor após a descongelação.

UNITERMOS: Etilenoglicol; Crioprotetores; Congelamento; Sêmen; Cães.

REFERENCES

1- ANDERSEN, K. Insemination with frozen semen based on a new insemination technique. **Zuchthygiene**, v.10, n.1, p.1-4, 1975.

2- BELLUZI, S.; ZAMBELLI, D.; BERGONZONI, M.L. L'inseminazione artificiale nel cane con seme congelato. **Praxis Veterinaria**, v.14, n.2, p.12-5, 1993.

3- BLOM, E. The ultrastructure of some characteristic sperm defects and a proposal for a new classification of the bull spermiogram. *In*: VII Simp. Int. di Zoot, Milan, 1972. Anais, 1972. p.125-39.

4- CONCANNON, P.W.; BATTISTA, M. Canine semen freezing and artificial insemination. *In*: KIRK, R.W. **Current veterinary therapy X**. Philadelphia, WB Saunders, 1989. p.1247-59.

5- DOBRINSKI, I.; LULAIS, C.; BARTH, A.D.; POST, K. Effects of four different extenders and three different freezing rates on post-thaw viability of dog semen. **Journal of Reproduction and Fertility**, Suppl. 47, p.291-6, 1993.

6- ENGLAND, G.C.W.; ALLEN, W.E. Factors affecting the viability of canine spermatozoa. II. Effects of seminal plasma and blood. **Theriogenology**; v.37, n.2, p.373-81,1992.

7- HARRISON, R.A.P.; VICKERS, S.E Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. **Journal of Reproduction and Fertility**, v.88, n.1, p. 343-52, 1990.

8- JOHNSTON, S.D. Performing a complete canine semen evaluation in a small animal hospital. **Veterinary Clinics of North America**, v.21, n.3, p.545-51, 1991.

9- JOSHUA, J.O. Reproductive clinical problems in the dog, Bristol, John Wright, 1984. p.49-60.

10- LE GAL, F.; BARIL, G.; VALLET, J.C.; LEBOEUF, B. In vivo and in vitro survival of goat embryos after freezing with ethylene glycol or glycerol. **Theriogenology**, v.40, n.4, p.771-7, 1993.

11- LINDE-FORSBERG, C. Artificial insemination with fresh, chilled extended, and frozen-thawed semen in the dog. **Seminars of Veterinary Medicine and Surgery (Small Animal)**, v.10, n.1, p.48-58, 1995.

12- LINDE-FORSBERG, C. Achieving canine pregnancy by using frozen or chilled extended semen. **Veterinary Clinics of North America**, v.21, n.3, p.467-85, 1991.

13- LINDE-FORSBERG, C.; FORSBERG, M. Results of 527 controlled artificial inseminations in dogs. **Journal of Reproduction and Fertility**, Suppl. 47, p.313-23, 1993.

14- MAEDA, T.; TERADA, T.; TSUTSUMI, Y. Comparative study of the effects of various cryoprotectants in preserving the morphology of frozen and thawed fowl spermatozoa. **British Poultry Science**, v.25, p.547-53, 1984.

15- MERCK INDEX, An encyclopedia of chemicals and drugs. Rahway New Jersey, Merck, 1976. p.3736.

16- OLAR, T.T. Using frozen canine semen: a guide for practitioners. **Veterinary Medicine**, v.80, p.22-30, 1985.

17- RAO, A. **Changes in the morphology of sperm during their passage through the genital tract in bulls with normal and impaired spermatogenesis**. Thesis. Stocolmo, 1971.

18- SEAGER, S.W.J.; PLATZ, C.C. Collection and evaluation of canine semen. **Veterinary Clinics of North America**, v.7, n.4, p.765-73, 1977.

19- SEIDEL, G.E. Principles of cryopreservation of mammalian embryos. *In*: BOVINE EMBRYO TRANSFER WORKSHOP, 70., Sydney, 1984. **Proceedings**. Sydney, The post-graduate committee in veterinary science, 1984. p.107-14.

20- SMITH, F.O. Atualidades em congelamento de sêmen canino. *In*: KIRK, R.W. **Atualização terapêutica veterinária**. São Paulo : Manole, 1988. p.1567-73.

21- STEEL, R.G.C.; TORRIE, J.H. **Principles and procedures of statistics and biometrical approach**, New York : McGraw Hill, 1980. p.633.

22- SZÉLL, A.; SHELTON, J.N.; SZÉLL, K. Osmotic characteristics of sheep and cattle embryos. **Cryobiology**, v.26, p.297-301, 1989.

23- THOMAS, P.G.A.; LARSEN, R.E.; BURNS, J.M.; HAHN, C.N. A comparison of three packaging techniques using two extenders for the cryopreservation of canine semen. **Theriogenology**, v.40, n.6, p.1199-205, 1993.

24- WILSON, M.S. Non-surgical intrauterine artificial insemination in bitches using frozen semen. **Journal of Reproduction and Fertility**, Suppl. 47, p.307-11, 1993.

25- YUBI, A.C.; FERGUNSON, J.M.; RENTON, J.P.; HARKER, S.; HARVEY, M.J.A.; BAGYENJI, B.; DOUGLAS, T.A. Some observation on the dilution, cooling and freezing of canine semen. **Journal of Small Animal Practice**, v.28, n.8, p.753-61, 1987.

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